Understanding the Biochemical Warfare in the Nests of Fungus-Growing Ants

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Abstract

Antimicrobial resistance (AMR) is a growing health concern, estimated to cause 1.27 million deaths worldwide in 2019. New antimicrobials are required to improve treatment options that evade resistance. However, the rediscovery of previously-reported antimicrobials has become a common occurrence. One way to reduce the risk of rediscovery is to search for bacteria producing natural products in under-explored environmental niches. Fungus-growing ants cultivate a fungal garden, consumed by the colony as a food source. The specialised pathogen, *Escovopsis*, can invade these fungal gardens and, if left uncontrolled, cause colony collapse. To prevent this, fungus-growing ants have formed a symbiotic relationship with antimicrobial-producing actinomycete bacteria, most notably *Pseudonocardia*. Despite evolving this relationship approximately 50 million years ago, fungus-growing ant colonies show few AMR-associated problems.

Here, 18 actinomycetes isolated from colonies of fungus-growing ants across Africa and South America were assessed for their ability to inhibit *B. subtilis, E. coli* and *C. albicans* on 35 different growth media, and their genomes were analysed. These strains came from multiple genera, including *Streptomyces, Pseudonocardia, Amycolatopsis* and *Jiangella.* Chemical extractions of three strains, which demonstrated antimicrobial activity but did not contain an antimicrobial-encoding biosynthetic gene cluster (BGC), were performed. Initial attempts were made to purify and identify the compounds responsible for antimicrobial activity via biological activity-guided fractionation. Further, ex-conjugants of two strains were generated using CRISPR/Cas9 to disrupt BGCs potentially encoding antimicrobials, in one strain leading to the cessation of antifungal activity. Initial attempts at comparative metabolomics were performed to identify the antifungal compound.

Although no novel antimicrobial was uncovered, the analysis of the 18 actinomycete strains showed that fungus-growing ant colonies are a reservoir of unreported secondary metabolites. Further, this work demonstrated the ability of the pCRISPomyces-2 system to be applicable to environmental *Streptomyces* strains, allowing for further studies in these strains to be performed.

This thesis is 220 pages and 61,420 words in length

Publications arising from this work in this thesis

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Contents

1 Introduction

1.1 Antibiotics

The discovery of the first 'modern' antibiotics to treat and prevent disease revolutionised healthcare in the first half of the 20th century and has had a lasting impact on human history. Antibiotic compounds are antimicrobials that exploit the differences between bacterial and eukaryotic cells to inhibit the growth of or kill bacterial cells whilst minimising side effects to the host organism. This means antibiotics are unusual therapeutics that assist in curing the disease instead of merely mitigating symptoms. The mechanism of action of antibiotics can include targeting enzymes in biosynthetic pathways unique to bacteria (e.g. β-lactams targeting DD-transpeptidases and other penicillin-binding proteins that cross-link the peptidoglycan cell wall), inhibiting protein synthesis by binding to ribosomal subunits unique to prokaryotic organisms (e.g. aminoglycosides bind to the 30S ribosomal subunit and cause mistranslation of messenger RNA (mRNA) and macrolides bind to the 50S ribosomal subunit and inhibit the translocation of tRNA from the A- to P-site), or disrupting the cell membrane (e.g. lipopeptides that insert into the membrane and cause rapid depolarisation, potentially leading to inhibition of protein and DNA synthesis) (Sultan *et al.*, 2018). Almost all classes of antibiotics used in human and veterinary clinics today were identified in the 1940s and 50s – the so-called 'golden age' of antibiotic discovery (Bérdy, 2005). Since entering the antibiotic era, many previously lethal infections, including tuberculosis, typhoid fever and bacterial meningitis, have become treatable and survivable. Antibiotics have also allowed for more complex interventions and surgeries that would have previously carried a high risk of infection, such as organ transplants and caesarean sections, and reduced the risk of other non-surgical treatments, including chemotherapy and diabetes. Antibiotics have also improved survival rates for HIV-positive patients (Livermore, 2004).

Microorganisms must constantly adapt and evolve to survive in an ever-changing environment to compete for nutrients with other organisms, survive in extreme temperatures (Somayaji *et al.*, 2022), protect themselves from ionising radiation

(Dadachova *et al.*, 2007) and osmotic stress (Bremer and Krämer, 2019). Though expensive to produce, secondary metabolites can provide a competitive advantage for the producing bacterium over its neighbours (Hibbing et al., 2010). Secondary metabolites include a variety of molecules, including antibiotics, siderophores, quorum-sensing molecules and exotoxins. The first and most widely known example of a secondary metabolite used in human medicine for antimicrobial purposes is penicillin, which Alexander Fleming discovered in 1928. Penicillin is an antibacterial produced by the mould *Penicillium chrysogenum* (then known as *P. notatum* (Fleming, 1929)). Fleming's discovery, combined with work from Howard Florey, Ernst Chain and Edward Abraham, developed penicillin into the drug we know today, which heralded the beginning of the golden age of antibiotic discovery. Around 80% of the antibiotics discovered during this golden age and 50% of antibiotics and antifungals used in clinics today are derived from secondary metabolites produced by bacteria hailing from the Actinomycetales order (Bérdy, 2005; Van Der Heul et al., 2018). The discovery of actinomycin in 1940 from the soil-dwelling actinomycete, *Streptomyces antibioticus* (then known as *Actinomyces antibioticus*), led to the belief that actinomycetes could prove a valuable source of antimicrobial compounds (Waksman and Woodruff, 1941). Actinomycin, however, was found to be toxic to mammalian cells, and it was not until the discovery of the aminoglycoside antibiotic streptomycin in 1943 that a natural product from an actinomycete was well characterised and used in clinics – in this case, to treat tuberculosis (Ohnishi et al., 2008). Since then, actinomycetes, most notably the genus *Streptomyces*, have been responsible for many other chemotherapeutic agents, including chloramphenicol, tetracyclines, the erythromycins, novobiocin, nystatin, streptothricin and daptomycin (Mahajan and Balachandran, 2012). Since the 1940s, around 9000 bioactive compounds have been isolated from actinomycetes, of which approximately 60 are still used in clinics, agriculture or research today (Demain, 2009). However, from the 1960s, the pipeline of new antibiotics began to dry up as compound rediscovery became more common (Vikeli et al., 2020). This prompted a shift in research to more closely study the rare actinomycetes – actinomycetes whose isolation rates by conventional methods are significantly lower than that of *Streptomyces* species - as the problem of emerging disease and resistance to existing

chemotherapy options became more apparent. Between the 1970s and 1990s, the discovery of secondary metabolites from these rare actinomycetes increased by around 30%. Despite this, most of the antibiotics isolated in this period were structural alterations to existing scaffolds and pharmacophores rather than distinct classes (Bérdy, 2005; Genilloud, 2017).

Due in part to the problem of rediscovery, pharmaceutical companies from the 1970s began attempting to produce synthetic, rationally designed antibiotics rather than continuing their search for novel natural products. The lack of success with synthetically derived antibiotics is evidenced by 70 of the 90 antibiotics marketed between 1982 and 2002 being derived from natural products. Most of the remaining products belonging to the fluoroquinolone class are arguably only semi-synthetic as their origins go back to nalidixic acid and research to synthesise the natural antimalarial chloroquine (Newman, Cragg and Snader, 2003). Of the three new classes of antibiotics released onto the market between 1985 and 2009, streptogramins, oxazolidinones and the lipopeptide daptomycin, only one (linezolid) can be considered entirely synthetic (Conly and Johnston, 2005; Boucher et al., 2009). More recently, pharmaceutical companies realised that antibiotic discovery is not as cost-effective for their business model when compared to more lucrative disease treatments, for example, for cardiovascular conditions, oncological or respiratory diseases, which are needed by the patient for extended durations, unlike a single course of antibiotics, and effect more significant numbers of people globally (H. Wang *et al.*, 2016). Accordingly, only four large pharmaceutical companies (Merck & Co., Roche, GlaxoSmithKline and Pfizer) have active antibiotic programs, of which only GlaxoSmithKline and Pfizer have active clinical trials for anti-infectives currently (Nature Biotechnology, 2018). With a limited number of new antibiotics, no new classes of antibiotics being brought to market and resistance to existing treatments, bacterial infectious disease is once again becoming a challenge to healthcare systems worldwide, with 1.27 million deaths attributed to antimicrobial-resistant bacterial infections in 2019 (Murray et al., 2022).

1.2 Antimicrobial Resistance

Antimicrobial Resistance (AMR) is when a microorganism can withstand antimicrobial agent concentrations at which the host organism experiences acceptable side effects. This effectively means that the antimicrobial can no longer be used in a clinical setting to treat an infected patient for fear of harming the patient. An alternative antimicrobial must be used if one is available. However, some drug-resistant infections are now virtually untreatable, such as “super-gonorrhoea” caused by *Neisseria gonorrhoeae* with resistance to penicillin, cephalosporin, azithromycin, tetracycline, fluoroquinolone and cefixime – leaving limited effective treatment regimens (Deguchi et al., 2010; Pleininger et al., 2022). Such infections utilise more hospital resources and increase patient mortality. AMR is predicted to cause 10 million deaths annually by 2050 and cost an estimated 100 trillion United States Dollars’ worth of lost economic output (O’Neil, 2016). AMR is listed on the United Kingdom’s National Risk Register, alongside climate change, large-scale nuclear attack and extreme cold (Cabinet Office, 2017). The success of antimicrobial compounds led to their widespread use in clinical, veterinary and agricultural settings, with 34.8 billion defined daily doses of antibiotics used globally in 2015, an increase from 21.1 billion 10 years prior (Klein et al., 2018). This leads to antibiotics leaching into the environment from agri- and aqua-culture, where up to 75% of antibiotics ingested by livestock are excreted unmetabolised (Chee-Sanford et al., 2009). Alternatively, antibiotics can enter the environment via wastewater from domestic and hospital sources, due to human excretion or improper disposal of antimicrobial compounds, with wastewater treatment plants often unable to remove antibiotics (Zhang et al., 2017; Zorpas, Dimitriou and Voukkali, 2018). In addition, antibiotics are incorrectly disposed of into refuse sites, with the site’s now contaminated leachate flowing into groundwater (Song et al., 2016). These antibiotics cause selective pressure on bacteria in the environment to evolve or maintain genes conferring AMR (Gullberg et al., 2014). Despite this, it must be remembered that various fungi and bacteria naturally produce antibiotics as part of their usual selection of secondary metabolites, which must also be resistant to them to prevent adverse effects on their metabolism. This means that the resistance genes

associated with any antibiotic are already present in the environment, in the organism that produces the antimicrobial compound and potentially in organisms surrounding the producer, as they compete for resources (Davis and Davis, 2010). AMR genes have been found to be widespread in the environment, with all 71 environments sampled in a 2014 global meta-genome study containing genes conferring AMR (Nesme et al., 2014). Other studies have found genes conferring AMR in bacteria frozen in arctic soil approximately 5000 years ago (Perron et al., 2015) and in an isolated cave microbiome (Bhullar et al., 2012).

One of the main drivers of the increase in AMR is the misuse of antibiotics. Around 40% of patients are not compliant with their antibiotic prescription (Kardas et al., 2005). Treatment with antibiotics is generally effective at removing community-acquired infections. However, by cutting short treatment, there is a risk that, while the treatment has removed susceptible isolates, isolates resistant to the front-line antibiotic remain to colonise the host after premature cessation of treatment (Ventola, 2015). Additionally, over 20% of antibiotic prescriptions in the UK are inappropriate, being prescribed for respiratory infections like sore throats and coughs, which are likely to be caused by viral infections and often do not need treatment beyond the relief of symptoms (Smieszek et al., 2018). Worse, from an antibiotic stewardship position, is that antibiotics are available over the counter or online without a prescription in many countries. In developing countries, people may not be able to afford an entire course of antibiotics, so they may only buy one or two tablets if they feel unwell without consultation with a trained healthcare provider (Ayukekbong, Ntemgwa and Atabe, 2017). On top of this, prescribing physicians often have little information on the exact nature of the causative agent of infection, leading to inappropriate or broad-spectrum antibiotics being prescribed where a more specific antibiotic may be appropriate. This is due to a lack of rapid diagnostic equipment available at the point of care and, whilst it may be the optimal pathway for patient care, drives the development of AMR.

Bacteria can evolve or acquire a variety of mechanisms to resist chemotoxic compounds. Drug-efflux pumps, including the Mex and Tol class pumps, can efflux multiple drugs from the cell and prevent their accumulation (Webber and Piddock,

2003). Alternatively, the antibiotic may be degraded enzymatically, for example, by TEM-1 and SHV-1 extended-spectrum β-lactamase (ESBL) enzymes (Livermore, 2008), or the target of the drug may be protected, demonstrated by the *tet(M)* and *otr(A)* genes in Gram-positive bacteria that encode for a protein with an elongation factor-like activity, stabilising the ribosome-tRNA complex during nucleotide translation when tetracycline is present (Chopra and Roberts, 2001). These adaptations can occur spontaneously through mutations or can be present on mobile genetic elements, such as plasmids, which can be distributed between bacteria by horizontal gene transfer. This ability to share genetic material, combined with the high reproduction rate of bacteria, allows them to rapidly evolve resistance to antibiotics, more rapidly than new antibiotics can be discovered and brought to market (Ventola, 2015).

Pathogenic bacteria acquiring and disseminating AMR genes compromise the ability of our current antibiotics to treat patients. This results in an inability to treat infections caused by drug-resistant strains of bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, which have demonstrated resistance to a wide range of antibiotics. Of particular concern is the emergence of the plasmid-mediated MCR-1 gene in *E. coli,* a transferable gene which confers resistance to colistin, a 'last resort' polymyxin class antibiotic used to treat Gram-negative carbapenemase producers and may lead to pan-resistant bacteria (Caniaux et al., 2017). Drug-resistant Gram-positive bacteria are also of significant concern. *Staphylococcus aureus* is a commensal bacterium that is a leading cause of skin and soft tissue infections and nosocomial acquired infections (Turner et al., 2019). Methicillin-resistant *S. aureus* (MRSA) accounts for approximately 60% of *S. aureus* infections in intensive care units and caused over 14,000 deaths in England and Wales between 1993 and 2012 (Rice, 2006; Office for National Statistics, 2013). Vancomycin-resistant *Enterococcus* (VRE) is another Gram-positive pathogen, first observed in the 1980s. VRE now makes up nearly 30% of *enterococci* infections in intensive care units (Rice, 2006). *Enterococci* are intrinsically resistant to many classes of antibiotics due to the penicillin-binding proteins they express having a low affinity for β-lactams and cephalosporins (Hollenbeck and Rice, 2012); they also express the *lsa* gene, an (ABC)-efflux pump

that effluxes lincosamide and streptogramin class antibiotics (Singh, Weinstock and Murray, 2002) and can prevent aminoglycoside permeating into the cell (Zimmermann, Moellering and Weinberg, 1971). Vancomycin, a glycopeptide class antibiotic initially discovered in the rare actinomycete *Amycolatopsis orientalis* (formally *S. orientalis*), is the first-line antibiotic for *Enterococci* infection (Levine, 2006). Glycopeptides inhibit bacterial cell wall synthesis by binding to the terminal D-alanyl-D-alanine in the stem peptides of cell wall precursors. Thus, they inhibit transpeptidase activity and cross-linking of the peptidoglycan layer, increasing its permeability and leading to cell death. Resistance is conferred primarily by altering the target to D-alanyl-D-lactate, in the case of *VanA*, B and D resistance genes, or to D-alanyl-D-serine, in the case of *VanC*, *E* and *G* (Levine, 2006). This resistance leaves linezolid and daptomycin as the primary treatments for VRE, both of which came with a high likelihood of side effects, and neither are suitable for use in pregnant women (National Institute for Health and Care Excellence, 2020b, 2020a). Unfortunately, AMR is not limited to bacterial infections. Fungi from the genus *Candida*, and in particular *Candida albicans,* have been showing an increase in resistance to azoles and echinocandins, the two main classes of antifungals used to treat *Candida* infection (Pristov and Ghannoum, 2019). Additionally, emerging fungal opportunists, such as *Scedosporium* species and *Lomentospora prolificans* (formally *Scedosporium prolificans*), demonstrate low levels of susceptibility to current antifungal drugs, with *L. prolificans* demonstrating pan-drug resistance (Pellon et al., 2018; Ramirez-Garcia et al., 2018). The spread of resistance amongst pathogens shows the urgent importance of identifying new antimicrobials to treat infectious disease.

1.3 Natural Products as Antibiotics

As synthetically designed antibiotics have largely not proven effective, research emphasis has returned to natural products to discover new compounds. This return to natural sources may also lead to an increased chance of discovering compounds with novel mechanisms of action, as rationally designed compounds often rely on screening chemical structures limited to 500Da in size, per Lipinski rules for drug design, smaller than many effective antibiotics derived from natural products (Lewis, 2017). Obtaining antibiotic candidates from natural products also has the advantage

of leveraging the millions of years of evolution, ensuring that the producing organism gains the most advantage from its secondary metabolites. As such, natural products are often more effective at crossing the membrane of prokaryotes and interacting with intracellular machinery than synthetically designed structures (Stone and Williams, 1992). Although some smaller molecules, including terpenes and alkaloids, have been shown to demonstrate antimicrobial properties, most antimicrobial natural products often have many reactive functional groups, multiple chiral centres and concatenated rings. These features require complex enzymatic pathways and multi-enzyme complexes such as polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) to be synthesised (Verdine, 1996; Demain, 2009). Almost exclusively, the genes encoding for the enzymes involved in the biosynthetic pathway of these complex secondary metabolites are clustered together in the genome of the producing organism in biosynthetic gene clusters (BGC), allowing for better regulation of secondary metabolite synthesis and easier transmission, vertically and horizontally, between bacteria (Jensen, 2016). These BGCs can be over 100Kb in size and contain little or no genetic information that is not directly related to the synthesis, regulation, resistance to or transport of the secondary metabolite they encode (Bibb, 2005). In addition to these relatively easy-to-identify PKS and NRPS BGCs, actinomycetes produce ribosomally synthesised and post-translationally modified peptides (RiPP). Some RiPPs have also exhibited antimicrobial activity (Devine, Hutchings and Holmes, 2017).

1.3.1 Non-Ribosomal Peptide Synthetases

NRPS are complex, multifunctional mega-enzymes that assemble complex secondary metabolites in bacteria and fungi by incorporating amino acids using C-N bonds. The non-ribosomal peptides (NRP) assembled by NRPSs can contain unique structural features, including heteroatomic rings, D-amino acids, and *N*-methylated residues, which may then be modified by tailoring enzymes incorporating fatty acids or sugars to produce more structural diversity and effect bioactivity (Sieber and Marahiel, 2005). NRPSs contrast ribosomally produced proteins by incorporating building blocks beyond the usual 20 amino acids and producing products independent of the presence of nucleic acid, for example, in the synthesis of the antibacterial peptide

gramicidin S (Lipmann et al., 1971). Many antibiotics and antifungal agents produced by NRPSs, including vancomycin, fengucin and pristinamycin, contain high levels of cyclisation or side chain cross-linking, constraining the molecule in its biologically active conformation. NRPSs can be classified into three groups – type A, B and C. Regardless of type, a typical NRPS catalytic domain – or module - at a minimum contains an adenylation (A) domain for amino acid recognition, a condensation (C) domain for amino acid activation and polymerisation and, a thiolation (T) domain, also known as a peptidyl carrier protein (PCP), which tethers the amino acid building block and peptidyl intermediate as they are modified by the other domains of the NRPS module, as shown in Figure 1 (Hur, Vickery and Burkart, 2012). The initiation and termination modules are an exception; the former lacks a C domain, and the latter processes a thioesterase (TE) domain, which releases the peptide by either hydrolysis or intramolecular macrocyclization (Horsman, Hari and Boddy, 2016). Some modules may also contain other domains responsible for a variety of modifications, for example, the epimerase (E) domain for inverse stereochemistry, the cyclisation (Cy) domain to form ring structures and the methyltransferase (MT) domain to methylate functional groups (Schneider and Marahiel, 1998). E domains allow for the conversion of L-amino acids into D-amino acids. The presence of D-amino acids alters the steric properties of the NRP, allowing for proper modification by downstream enzymes, in particular condensation reactions (Rausch et al., 2007). Cy domains are often specialised versions of C domains, simultaneously condensing and cyclising the peptide chain. In this cyclisation role, Cy domains most commonly act upon cysteine or serine amino acids to form ring structures within the NRP, allowing for more significant structural diversity and consistency in relative positions of functional groups required for bioactivity (Pang, Wang and Liu, 2016). MT domains catalyse the transfer of the S-methyl group of S-adenosyl methionine to the α-amino group of the amino acid directly attached to the PCP, allowing for the formation of methylated-N domains, for example, in the biosynthesis of actinomycin D by *S. parvullus* (Sieber and Marahiel, 2005).

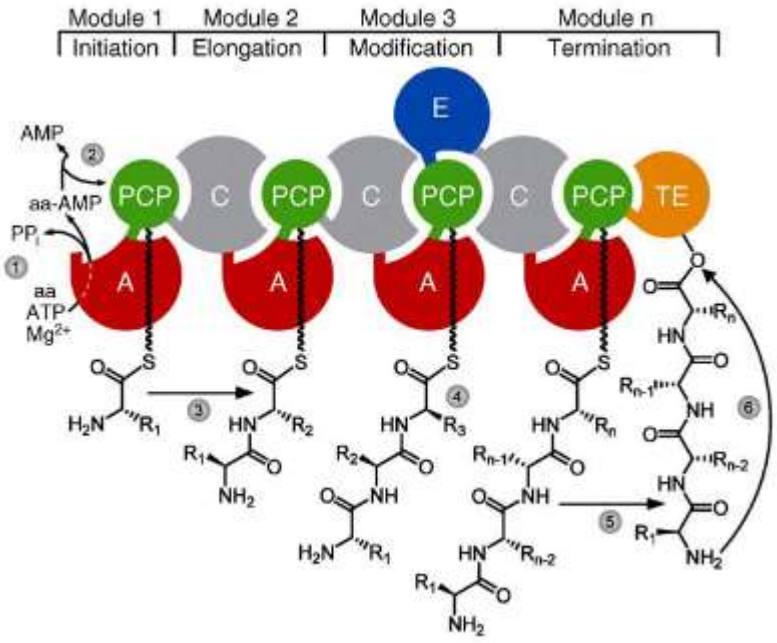


Figure 1 – Structure of a basic NRPS module. 1) The amino acid is activated in the A-domain. 2) The amino acid is then transferred to the PCP in the T-domain. 3) Condensation of PCP-bound amino acid occurs in the C-domain. 4) The amino acid may then be modified in some modules. 5) The PCP transfers the peptide by transesterification onto the TE domain. 6) The product is hydrolysed or macrocyclised to release it. Image adapted from (Strieker, Tanović and Marahiel, 2010)

Type A NRPSs direct biosynthesis of NRPs linearly, and as such, the number and sequence of modules in a type A NRPS closely matches the number and order of amino acids in the NRP. Glycopeptide antibiotics like vancomycin are usually synthesised by type A NRPSs. Type B NRPS synthesise NRPs in an iterative manner, with some modules used multiple times. For example, the biosynthesis quinoxaline family of natural products, including echinomycin and the veterinary antibiotic carbadox (Felnagle et al., 2008). Chemicals of the quinoxaline family bind to duplex DNA by bisintercalation, inserting two planar chromophores between base pairs, placing its cyclic backbone in the minor groove, thus disrupting DNA polymerase activity (Dawson et al., 2007). The chromophore can be decorated with hydroxyl and methoxy groups by tailoring enzymes, altering the activity of the quinoxaline. Echinomycin has two-fold symmetry in the molecule, consisting of two copies of a five-residue peptide condensed into a cyclic product, as illustrated in Figure 2; this requires repeated use of module 5 to dimerise the two monomers.

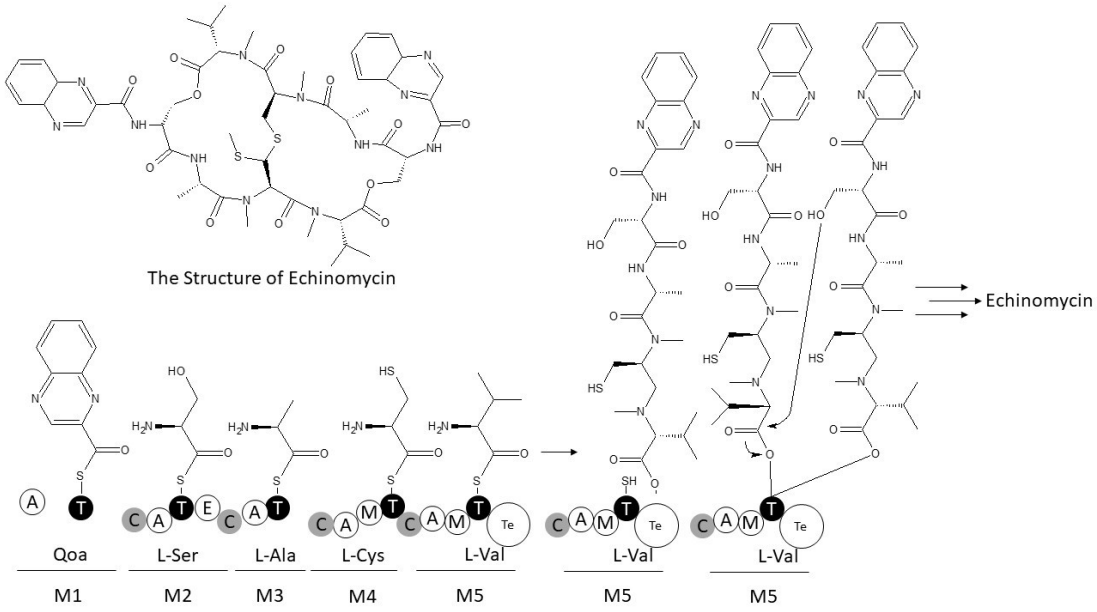


Figure 2 – **The Biosynthetic Pathway of Echinomycin Using a Type B NRPS**. Module 5 (M5) is used multiple times to assemble the final product. Enzyme key: A – adenylation domain, T – thiolation domain, C – condensation domain, E – epimerase, M – methyltransferase, TE – Thioesterase. Adapted from (Felnagle *et al.*, 2008)

In type C NRPS, the natural product is synthesised non-linearly. As such, module order is a poor predictor of NRP structure as the sequence of amino acids does not correlate to the arrangement or quantity of modules in the NRPS. Capreomycin is used in conjunction with other antibiotics to treat multi-drug resistant (MDR) *Mycobacterium tuberculosis*, targeting 16S and 23S rRNA (Johansen et al., 2006). The pharmacophore of capreomycin includes four nonproteinogenic amino acids and one proteinogenic amino acid. Despite containing five amino acids, the NRPS for capreomycin consists of only four modules containing an A domain - CmnF, CmnA, CmnI and CmnG – the fifth module – CmnJ - is an acyl-CoA dehydrogenase homolog possessing no A site for amino acid activation (Felnagle et al., 2007).

Recent discoveries of NRPS synthesised antibiotics with novel mechanisms of action demonstrate the value of continuing research on NRPs. Teixobactin was discovered by Ling and colleagues (2015) using an iChip, a device designed to isolate and culture bacteria that are unculturable using traditional methods, which are believed to account for around 99% of soil microorganisms (Pham and Kim, 2012). The iChip allows in situ bacterial cultivation by diluting a soil sample so that each channel in the iChip receives, on average, a single bacterial cell. These are then covered by two

semi-permeable membranes and submerged back into the soil from which the samples were taken, allowing for diffusion of nutrients, growth factors and chemical signals from surrounding organisms (Nichols et al., 2010). This allowed for the isolation and cultivation of the provisionally named *Eleftheria terrae,* a new species of β-proteobacteria, of a new genus related to *Aquabacteria*, a genus of Gram-negative organisms not known to be significant producers of antibiotics (Ling et al., 2015). Teixobactin contains seven L- and four D-amino acid residues and two ester linkages, making it a depsipeptide, encoded by 11 NRPS modules across 2 NRPS genes *txo1* and *txo2* (Guo et al., 2018). Teixobactin demonstrates activity against drug-resistant Gram-positive bacteria, including *enterococci*, *M. tuberculosis* and *Clostridium difficle*, but it has minimal activity against Gram-negative bacteria. The activity is caused by teixobactin's ability to bind to two precursors of bacteria cell-wall polymer: lipid II (peptidoglycan) and lipid III (teichoic acid), which explains the efficacy of teixobactin against Gram-positive bacteria, that possess thick peptidoglycan layers in their teichoic acid-containing cell wall. This also explains the lack of efficacy against Gram-negative bacteria, where the outer membrane prevents access to lipid II and lacks teichoic acid (Ling et al., 2015; Wright, 2015). This is further evidenced by resistance to teixobactin not being observed after culturing *S. aureus* and *M. tuberculosis* with a sub-minimum inhibitory concentration (MIC) of teixobactin, suggesting an endogenous protein is not targeted as it is relatively easy for a mutation in the encoding gene to convey resistance (Ling et al., 2015; Guo et al., 2018).

1.3.2 Polyketide Synthases

PKS gene clusters, similarly to NRPS gene clusters, are multifunctional enzyme structures responsible for a diverse range of natural products, including many antimicrobials such as the antibiotic erythromycin A – visualised in **Figure 3** - and the antifungal amphotericin B (Hopwood, 2007; Robbins et al., 2016). Both are multi-modular enzymatic arrays acting in an assembly line fashion. The key difference is that PKSs utilise organic acids, primarily malonyl-Coenzyme A (CoA) or methyl malonyl-CoA, as monomeric building blocks as opposed to the amino acids preferred by NRPSs. PKSs are divided into types I, II and III, with type I further divided into

noniterative and modular, although noniterative type I PKS (T1PKS) are primarily found in fungi so will not be discussed further here (Hopwood, 1997; Moore and Hopke, 2001; Yu et al., 2012). A modular T1PKS polymerises malonyl-CoA and methyl malonyl-CoA monomers in a series of successive decarboxylative Claisen condensation reactions, resulting in a two carbon extension to the growing polyketide chain with each completed reaction (Ray and Moore, 2016). Each Claisen condensation occurs in a distinct catalytic domain before the chain is moved to the next domain. Like in NRPSs, these domains are also known as modules. These modules comprise, at a minimum, a ketosynthase (KS), an acyltransferase (AT) and an acyl-carrier protein (ACP) domain but may contain further tailoring enzymes, allowing for more complex structures to be assembled (Robbins et al., 2016; Moretto et al., 2019). The first module in a modular type I PKS is instead a loading module, containing only an AT and an ACP, which loads the starter unit onto the PKS. Starter units for modular T1PKSs are most commonly acetate-CoA or propionate-CoA, although other starter units, such as amino-acid derived short branched-chain carbonyl-CoAs including isobutyryl-CoA and 2-methylbutyryl, have been observed (Moore and Hertweck, 2002). The starter unit is bound by the loading AT and transferred to the loading ACP, allowing transfer to the KS in module 1. The ACP in module 1 is then loaded and shuttles the starting unit between the active sites in module 1 before handing it over to the ACP in module 2. After the loading modules, the ACP will usually visit an AT domain to load the following monomer and a KS domain to catalyse the Claisen condensation, extending the chain. The ACP may shuttle the growing chain to a ketoreductase (KR) to reduce the β-ketone to an alcohol, dehydratase (DH), to eliminate the β-hydroxyl group to form a C-C double bond, or enoylreductase (ER), to fully reduce the keto group to a β-methylene group (Moretto et al., 2019). The ACP domain will then pass the substrate onto the KS domain of the subsequent modules or release it with a thioesterase (TE). The 6-deoxyerthronolide B (6-dEB) macrolide core of erythromycin A, produced by *Saccharopolyspora erythraea* (formerly *S. erythraea*), is an example of a clinically-important molecule synthesised by a modular type I PKS. 6-dEB is assembled from one propionyl-CoA starter unit, to which six methyl malonyl-CoA units are added in turn using AT and KS. Between some chain extensions, KR, DH and ER subunits reduce

the β-keto group, as described above, before the TE releases the completed 6-dEB ring for oxidation and glycosylation tailoring by cytochrome p450 enzymes (Hopwood, 1997; Staunton and Wilkinson, 1997; Rawlings, 2001).

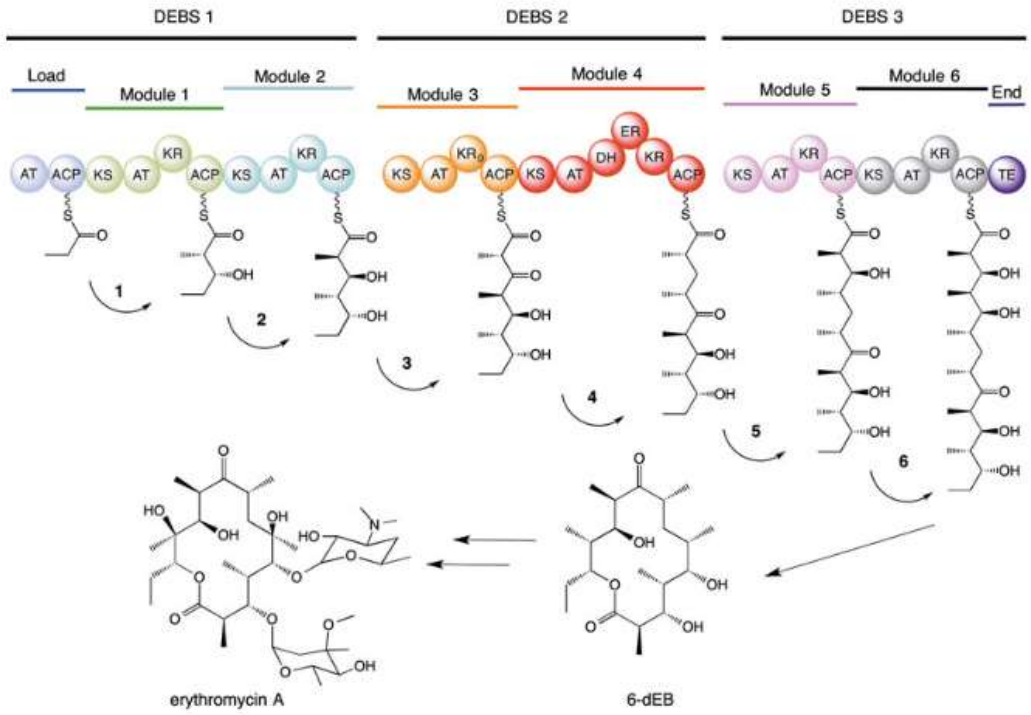


Figure 3 - The Biosynthetic Pathway of Erythromycin A using the Modular T1PKS 6-deoxyerthronolide B synthetase (DEBS). The numbered arrows indicate the order of the product chain extension and transfer, with each module coloured separately. Image adapted from (Till and Race, 2016).

Type II PKSs (T2PKS) contain similar catalytic domains as T1PKS. However, there are typically two KS domains, KSα and KSβ, instead of the single KS domain observed in T1PKSs. KSα is the T2PKS equivalent to the T1PKS KS domain, catalysing the Claisen condensation reaction responsible for chain extension, whilst KSβ controls the polyketide length of the polyketide chain (Chan et al., 2009). A 'minimal T2PKS PKS' therefore consists of the KSα and KSβ domains alongside an ACP to anchor the growing peptide chain. However, most contain additional ketoreductases, cyclases and aromatases, which act on the resulting poly-β-keto intermediate to generate a range of polyphenolics. This can then be tailored by oxygenases, methyltransferases, glycosyltransferases and halogenates to produce a diverse range of polyphenolic polyketides (Risdian, Mozef and Wink, 2019). Structural predictions of the products of T2PKSs are more complex than predictions of T1PKS products due to the former

relying on multiple enzymes rather than a production line style linear progression of the latter. Furthermore, the poly-β-keto intermediates are highly unstable and prone to spontaneous cyclization, making them difficult to isolate in order to aid understanding of the biosynthetic machinery (Hertweck et al., 2007).

Actinomycetes are prolific producers of secondary metabolites using T2PKSs. For example, in the production of benzoisochromanequinone class antibiotics such as medermycin by *Streptomyces sp.* K73 or the blue-pigmented actinorhodin, produced by the highly characterised model organism *S. coelicolor* A3(2) (Bentley et al., 2002; Risdian, Mozef and Wink, 2019). The *S. coelicolor* A3(2) actinorhodin BGC was one of the first smBGCs cloned and heterologously expressed in another organism to produce the first hybrid antibiotic and now is one of the most studied secondary metabolites produced by *Streptomyces* species (Hopwood et al., 1985). The carbon skeleton of actinorhodin is assembled using minimal PKS modules, containing only one each of KSα, KSβ and ACP, which polymerise eight malonate units to form an octaketide chain and then perform a series of cyclisations before tailoring enzymes finalise the structure (Beltran-Alvarez et al., 2007).

Type III PKSs (T3PKS) are self-contained enzymes that usually form homodimeric ring structures, such as phloroglucinols and chalcones (Yu et al., 2012). The first reported bacterial T3PKS was RppA in *S. griseus*, responsible for catalysing the synthesis of 1,3,6,8-tetrahydroxynaphthalene, a precursor of hexahydroxyperylenequinone melanin, a dark-brown pigment, from five molecules of malonyl-CoA (Funa et al., 2005). Later studies have found RppA-like enzymes in other actinomycetes, including *Saccharopolyospora erythrea* (Cortés et al., 2002) and *S. coelicolor* (Li et al., 2007). Although useful as precursors to other products, both in biosynthetic and synthetic pathways, the products created by T3PKSs are rarely bioactive and are generally used as substrates in further reactions rather than as active products in their own right (Yu et al., 2012).

1.3.3 Ribosomally Synthesised and Post-Translationally Modified Peptides

RiPPs are proteins with a molecular weight of less than 10kDa produced by the ribosome and undergo post-translational modification by tailoring enzymes (Arnison

et al., 2013). Interest in RiPPs has increased over the last several years as genome sequencing has become less expensive and more widely available because the chemical structures of RiPPs are highly predictable from genomic data. Bacteriocins were the first RiPPs to be identified. They form a diverse family of small peptides produced by nearly all bacteria, which are heat-stable and sensitive to proteases, meaning they are mostly harmless to humans and the environment. This has allowed for bacteriocins to be used as antibiotics, oncological drugs and food preservatives (Drider et al., 2016). As antibiotics, bacteriocins have high potency and can be both broad- and narrow-spectrum (McIntosh, Donia and Schmidt, 2009). Although resistance can develop to bacteriocins, using narrow-spectrum agents where possible will reduce the rate at which resistance emerges. As the bacteriocins used possess different mechanisms of action to traditional antibiotics, they can be used to treat otherwise drug-resistant infections. These mechanisms include DNA and RNA disruption or, more commonly, increasing the permeability of the cell membrane by targeting lipid II or forming pores in the cell wall (Cotter, Ross and Hill, 2013). Lantibiotics are another example of RiPPs with an antimicrobial function, defined by posttranslational additions of lanthionine, methyllanthionine, or both, from which they derive their name (lanthionine-containing antibiotic). Lantibiotics are generally only 19-38 amino acids in length, and all involve the dehydration of Ser and Thr residues followed by an intramolecular addition reaction to form a lanthionine or methyllanthionine bridge (Willey and van der Donk, 2007). Many lantibiotics have shown potent activity against MRSA and VRE, including nisin shown in Figure 4, the only commercially used lantibiotic, which has been used as a food preservative for 40 years, with work being done to expand it into food packaging and clinical use (Cotter, Hill and Ross, 2005; Willey and van der Donk, 2007).

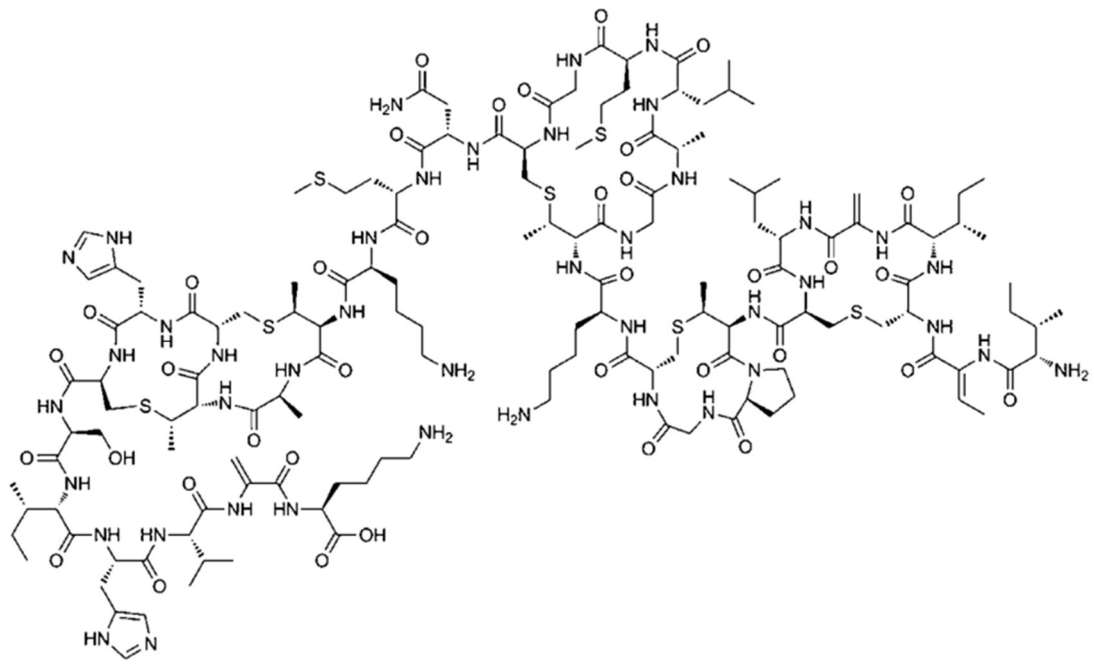


Figure 4 - The Chemical Structure of Nisin, an Antibiotic Encoded by a RiPP

Despite the broad range of structural classes RiPPs fall into, they all follow a similar biosynthetic pathway. A precursor peptide, typically 20-110 residues in length, consisting of an N-terminal leader and C-terminal core sequence is translated. Peptidases remove the leader sequence, and other enzymes modify the remaining active peptide. All relevant genes are encoded in a distinct cluster on the genome, similar to PKS and NRPS clusters described previously (Arnison et al., 2013).

1.4 Actinomycetes

Genes encoding biosynthetic machinery required to produce a variety of secondary metabolites are found across all domains of life. Amongst microorganisms, the Actinomycetales are amongst the most well-known for their ability to produce a vast range and quantity of secondary metabolites, including several important classes of antibiotics such as β-lactams, tetracyclines and rifamycins (Genilloud, 2017). Actinomycetales are an order of Actinobacteria (recently proposed to be renamed Actinomycetota (Oren and Garrity, 2021)) containing a diverse range of families, including; *Jiangellineae*, *Propionibacterineae* and *Streptomycineae*. A member of the Actinomycetales order is commonly called an actinomycete. Actinomycetes are usually Gram-positive, aerobic bacteria and can be found almost ubiquitously across

terrestrial and aquatic environments, including sea sponges, soil systems and insect colonies (Abdelmohsen et al., 2010; Bhatti, Haq and Bhat, 2017; Chevrette et al., 2019). Filamentous actinomycetes are unusual bacteria in that they form a filamentous multicellular mycelium, which led to them being confused with fungi when they were first discovered. In 2002, Bentley and colleagues published the complete genome sequence of the model actinomycete *S. coelicolor*, which was revealed to contain approximately 32 BGCs encoding for the biosynthesis of secondary metabolites (Van Keulen and Dyson, 2014). Despite being studied since the 1960s, only five smBGCs within *S. coelicolor* had been characterised in detail by the time the full genome sequence was published. This revelation defeated the 'new bugs, new drugs' and high-throughput screening based research ideologies that had been the mainstream view throughout the 1980s and 1990s and opened' genome mining' as a new method of identifying novel secondary metabolites from already isolated strains of actinomycete (Ward and Allenby, 2018). Advances in genome sequencing and the development of smBGC prediction tools such as antiSMASH have revealed that this experience is common to many actinomycetes, with only a fraction of actinomycete secondary metabolites produced under laboratory conditions and some genera such as the *Streptomyces*, *Micromonospora* and *Saccharopolyspora* dedicating over 5% of their genome to secondary metabolite production, often able to produce over 30 secondary metabolites (Nett, Ikeda and Moore, 2009; Blin et al., 2019). Genome mining now covers a diverse range of methodologies to identify the secondary metabolites encoded by these 'silent' or 'cryptic' secondary metabolite BGCs (smBGCs), including; 'trial and error' pleiotropic approaches to elicit expression, the prediction of chemical structures using the genetic information encoding for the biosynthetic machinery and, heterologous expression of biosynthetic pathways that cannot be elicited in their host organism (Bachmann, Van Lanen and Baltz, 2014). These methods are limited however. For example, the prediction of chemical structures analyses databases of highly-conserved biosynthetic enzymes, the results of which are then analysed using pre-defined but manually curated rules. This may lead to bias in the dataset, if particular enzyme types have been comparatively over-studied, or if the pre-defined rules are bias towards re-discovery by, for example, requiring a very high level of homology

between the cluster being investigated and the database. This may mean searches miss smBGCs encoding novel antimicrobial agents, with poor homology or similarity to previously discovered antimicrobials, or to antimicrobials where the genes encoding the enzymes responsible for its biosynthesis are spread throughout the genome (Ziemert, Alanjary and Weber, 2016). Meanwhile, heterologous expression requires the antimicrobial to not inhibit the growth of the organism being used to express it, and for that organism to have the appropriate biosynthetic machinery for the production to occur. Heterologous expression also rests on the assumption that all of the genes encoding the production of the antimicrobial are clustered, otherwise only some of the biosynthetic machinery required to product the antimicrobial will be expressed.

1.4.1 The *Streptomyces* Genus

Streptomyces are filamentous actinomycetes that represent the best-characterised genus of the Actinobacteria. They are responsible for around 80% of bioactive molecules known to be produced by Actinobacteria with a diverse range of functions, from antimicrobial to signalling, allowing them significant influence over their local environment (Demain, 2009). They are widespread in terrestrial and aquatic environments, and can be found in symbiotic relationships with a variety of plants and insects, including crop plants such as wheat, where the secondary metabolites *Streptomyces* produce help to protect the plant from disease and assist nitrogen fixation, granting them vast economic and ecological importance (Seipke, Kaltenpoth and Hutchings, 2012; Viaene et al., 2016). *Streptomyces* contain an average of 32.5 smBGCs, with up to 53 in some strains (Ward and Allenby, 2018). Despite intense study since the golden age of antibiotic discovery, the majority of *Streptomyces* BGCs are cryptic under laboratory conditions and could remain an untapped source of antibiotic agents. In order to 'unlock' these cryptic BGCs and discover the chemicals they encode, work must be performed to both encourage their activation under laboratory conditions and improve our understanding of the basic biology of *Streptomyces*, including the regulation and function of different secondary metabolites, including how these molecules interact with the environment around the producing organism. By doing this, we may be able to induce the production of

otherwise cryptic BGCs and discover novel antimicrobial compounds (Bachmann, Van Lanen and Baltz, 2014).

1.4.1.1 The Biology of *Streptomyces*

S. coelicolor has been the model *Streptomyces* organism for the past 60 years, with much of our understanding of the unusual *Streptomyces* lifecycle coming from studying this organism. More recently, studies have been conducted on *S.* griseus and *S. venezuelae,* which are now considered model organisms. As noted above, *Streptomyces* species are rare amongst bacteria in that they grow as a multi-cellular, filamentous network of vegetative hyphae that more closely resembles a fungal mycelium than a traditional bacterial colony (Hopwood, 2007). This filamentous network originates from a single spore, germinating into the highly-branched vegetative hyphae that extend by tip extension to scavenge for nutrients and are divided into compartments, speculated to limit the ability for phage to spread throughout the mycelium, as shown in Figure 5 (Chater, 2016). *Streptomyces* species are able to metabolise a wide range of insoluble carbon-rich polymers, such as chitin and lignocellulose, which are often found in soil as the remnants of dead organisms. Thus, *Streptomyces* species are important carbon recyclers in soil (Bentley et al., 2002). Vegetative hyphae then go on to form distinct tissue-like structures, similar to multicellular organisms. These can demonstrate division of labour, with some cells producing a higher quantity of a more diverse range of antibiotics whilst others form into aerial hyphae and form spores to restart the *Streptomyces* life-cycle, and others still are sacrificed through programmed cell death to provide nutrients for the remaining cells (McCormick and Flärdh, 2012; Z. Zhang *et al.*, 2020). These aerial hyphae emerge from vegetative mycelium coated by hydrophobic chaplin, rodlin and SapB proteins, which allows the aerial hyphae to break the surface tension of the aqueous environment and grow into the air (Kelemen and Buttner, 1998; Capstick et al., 2007). When the aerial hyphae form, chromosomal replication occurs before they are segregated into immature pre-spore cells, resulting in a uni-genomic chain of pre-spores forming from the aerial hyphae. When the appropriate cellular and environmental signals are received, these pre-spores will mature into thick-walled, often pigmented, spores that lie dormant until environmental conditions are

favourable for germination and growth (Bush et al., 2015). These spores are highly desiccant resistant, able to withstand the complex chemical ecology of the various environments *Streptomyces* species survive in, allowing the otherwise non-motile mycelium to spread into alternate geographical areas when exposed to nutritional or environmental stress (Bentley et al., 2002). In addition to the 'classic' growth cycle of *Streptomyces* bacteria, recent work by Jones and colleagues (2017) uncovered a previously unknown mode of bacterial growth known as 'exploration.' By co-culturing *Streptomyces* with yeast or on a glucose deficient medium, the mycelium was made to grow at significantly faster rates than under standard conditions (1.5µm/min when exploring, compared to 0.13µm/min under standard conditions), including over biotic surfaces, such as the co-cultured organism, and abiotic surfaces, such as plastic Petri dishes, glass coverslips and rocks. The exploring vegetative hyphae more closely resemble aerial hyphae than their non-exploring counterparts, having little branching although maintaining their hydrophilicity (Jones et al., 2017). The exploring hyphae have also been shown to induce exploring in other *Streptomyces* colonies through the production of the volatile organic compound trimethylamine. In this way, the otherwise non-motile *Streptomyces* is able to spread more efficiently into nearby environments to discover new nutrients without the need to commit to the terminal sporulation process (Jones and Elliot, 2017). In addition to the production of spores and the ability for exploration, *Streptomyces* species also use the secondary metabolites they produce to aid their survival in environments where they face fierce competition for space and nutrients from other microorganisms. Secondary metabolites are non-essential to growth, at least under laboratory conditions. These secondary metabolites have a variety of functions, including antimicrobial, and likely enable the producing organisms to better acquire local resources by inhibiting competitors, signalling between *Streptomyces* colonies or siderophores to enhance metal ion uptake (McCormick and Flärdh, 2012).

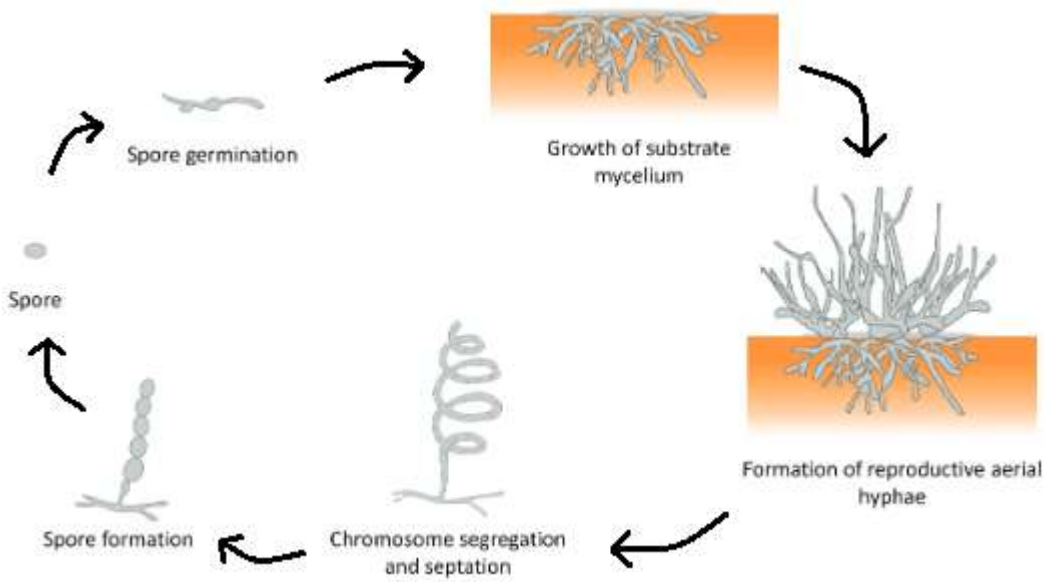


Figure 5 - The Life Cycle of *Streptomyces* adapted from (Law *et al.*, 2019) *Streptomyces* begin life as spores, which germinate when conditions are likely to be suitable for growth. Initially, substrate mycelium grows downwards into media. As the level of nutrients available decrease, aerial hyphae begin to form, growing upwards out of the medium before these segregating and forming spores.

Streptomyces species have large, linear chromosomes of 5-11Mb in length, capped by terminal inverted repeats (TIR) and have a GC content of 70% or higher (Hopwood, 2006; Harrison and Studholme, 2014). TIRs are palindromic sequences around 168 nucleotides in length, which protect the chromosome's terminus from damage and inhibit cyclisation (Goshi et al., 2002). A significant proportion of *Streptomyces* genetic material is dedicated to encoding for the biosynthetic machinery for secondary metabolite biosynthesis. These non-essential genes are generally located in the peripheral 'arms' of the chromosome, the 1-2Mb of DNA furthest from the centre, where the highly-conserved essential genes are located. *Streptomyces* species are genetically unstable and have a high rate of horizontal transfer of the genetic information in the arms, allowing smBGCs and regulatory elements to be shared between strains. These chromosome arms are, however, not suited to long-term retention of a gene and, as such, if a gene proves useful and is used repeatedly, it may migrate towards the central core of the chromosome that contains the essential genes (Chater and Chandra, 2006). This genetic instability leads to a high rate of mutations – often in more than 0.1% and up to 0.5% of colony-forming spores. These mutations can lead to morphological differentiation, increased production of

antibiotics or sometimes losing enzymes in primary metabolic pathways, although most mutants caused by this instability are hypervariable, displaying no alteration in phenotype. The majority of these mutant phenotypes are caused by deletions of genetic information, potentially up to 2Mb in length (Volff and Altenbuchner, 1998). These deletions can often lead to circularisation of the chromosome, caused by nonhomologous recombination between the left and right deletion ends (Nindita et al., 2013).

1.4.1.2 Regulation of *Streptomyces* Secondary Metabolite Production

Secondary metabolite production in *Streptomyces* species occurs throughout the life cycle, with the level of regulatory gene expression changing across growth stages for almost half of all *S. coelicolor* regulatory genes. Many of these genes are expressed at higher levels during the late exponential and stationary phase, as sporulation begins (Jeong *et al.*, 2016). Some of these regulatory genes encode sigma factors, proteins that enable bacterial transcription in response to environmental conditions by allowing RNA polymerases to bind to gene promotors. For example, $E\sigma^{hrdB}$, which regulates the production of actinodin and more generally mediates the relative production of secondary metabolites compared to primary, is more abundant during stationary phase and during sporulation than exponential phase (Kang *et al.*, 1997; Sun *et al.*, 2017). This effects the production of secondary metabolites, including the lantibiotic SapB (discussed in more detail in Section 3.3.10) and antibiotic actinorhodin being expressed at high levels during sporulation in *S. coelicolor* when compared to other growth stages (Jeong *et al.*, 2016). Other sigma factors, notably $E\sigma^{hrdD}$, which in part regulates undecylprodigiosin production and is activated in response to osmotic stress, are at higher concentrations in the exponential phase than stationary, however, and comparatively low levels during sporulation (Takano *et al.*, 1992; Sun *et al.*, 2017).

The sporulation process, an important part of the *Streptomyces* lifecycle is tightly regulated by the *bld* and *whi* families of regulators, although only the *bld* gene directly impacts secondary metabolite production. The *bld* gene family control a wide range of functions, most notably the erection of aerial hyphae, the name of the gene

family reflects this as mutants lacking the *bldB* gene appear 'bald' due to the lack of aerial hyphae (Hopwood, Wildermuth and Palmer, 1970). These *bld* genes have also been linked to the regulation of a variety of secondary metabolites, including antibiotics. For example, *BldD* is a DNA binding protein that acts as a master regulator, repressing approximately 170 genes, including 42 genes encoding for regulatory proteins, required for sporulation during vegetative growth. BldD achieves this by interacting with cyclic dimeric 3′-5′ guanosine monophosphate (c-di-GMP), a widespread secondary messenger among bacteria, and an important regulator in the *Streptomyces* lifecycle (Tschowri *et al.*, 2014; Latta and Bechthold, 2022). The BldD-c-di-GMP complex directly affects secondary metabolite synthesis by binding to promotor regions within BGCs and indirectly by repressing other bld regulators, such as *bldC*, which encodes for a DNA-binding protein that regulates gene transcription for many BGCs (Den Hengst et al., 2010). Additionally, *Streptomyces* mutants lacking the *bldA* gene, which encodes for a tRNA capable of translating a UUA codon into leucine, are not only unable to produce aerial hyphae and spores but were also deficient in antibiotic production when compared to the wild type. This is due to many secondary metabolite genes containing a TTA codon, which requires a functional *bldA* gene encoding leu-tRNAUUA to be translated. (Hackl and Bechthold, 2015). In *S. coelicolor,* BldA is directly involved in the upregulation of 147 genes during the stationary phase and aerial hyphae formation, although only two of these genes possess a TTA codon, suggesting that BldA is acting as a regulator indirectly. There are three possibilities as to how this could be occurring; firstly, is that the two TTA-containing genes are part of a signalling cascade triggered by BldA and go on to upregulate other genes; secondly is that mRNA containing a UUA codon have a reduced half-life and therefore are not translated into protein. Finally, mutants lacking BldA show increased levels of the signalling molecule guanosine tetraphosphate (ppGpp), usually released when under nutrient stress; changes in ppGpp concentration alter the transcription of ribosomal proteins, meaning BldA could be altering protein transcription via ppGpp (Hesketh et al., 2007). ppGpp is important in regulating secondary metabolite production as many secondary metabolite biosynthetic pathways utilise products or intermediates from primary metabolism. As nutrients such as carbon and nitrogen become more scarce,

Streptomyces species can divert metabolic pathways to increase amino acid and fatty acid synthesis and the concentration of ppGpp increases, which can activate antibiotic production to allow *Streptomyces* bacteria to compete with other organisms for the remaining resources (Bibb, 2005; Hesketh et al., 2007).

Many smBGCs are regulated by one or more localised cluster situated regulators (CSR), often under the control of the global regulators discussed previously (Bibb, 2005). *Streptomyces* antibiotic regulatory proteins (SARP) are the most common CSRs and include ActII-4 and RedD, responsible for regulating actinorhodin and undecylprodigiosin respectively. SARPs function either via direct transcriptional control of the biosynthetic genes in their cluster, as in the example of NosP in *S. actuosus* directly binding to the bidirectional NosL-M promotor region for nosiheptide biosynthesis, or by activating another CSR within the cluster, such as with the OrcR protein in *S. rimosus*, which activates oxytetracycline biosynthesis by binding to *oxyA, I, J, R* and *S* (S. Yin et al., 2015; Wu et al., 2018). Other SARPs function as repressors of antibiotic synthesis by directly inhibiting the transcription of cluster-situated activation genes. The ScbR regulator of coelimycin P1 biosynthesis, which inhibits the transcription of the SARP homologue encoding *kosO* in *S. coelicolor,* is one such example from the TetR family of transcriptional regulators (Wei, He and Niu, 2018). Regardless of how they function, all SARPs consist of a winged helix-turned-helix motif that binds to DNA, recognising the seven-nucleotide repeating units within the promotor region of the BGC (Aigle and Corre, 2012). Some SARPs, including TetR, are regulated in part by hormone-like signalling molecules, such as γ-butyrolactone, which is often an indicator of the presence of rival organisms (Niu et al., 2016). CSRs also regulate large ATP-binding regulators of the LuxR family (LAL) in actinomycetes. LALs range in size from 88 to 125kDa and contain a helix-turn-helix motif at their C-terminus, similar to SARPs, as well as an N-terminus ATP/GTP-binding domain, with the two terminals connected by a high-conserved core element (Schrijver and Mot, 1999). Although found in other bacteria, such as *Pseudomonas alcaligenesi*, the majority of LALs are found within the actinobacteria. Examples of LALs include PikD, which regulates pikromycin biosynthesis in *S. venezuelae* (Wilson et al., 2001) and nystatin biosynthesis in *S. noursei* regulated by NysRIV (Sekurova et

al., 2004). Multiple antibiotic resistance regulators (MarR), initially discovered in *E. coli*, are typically repressor CSRs found throughout bacteria as homodimers, with each monomer processing a winged helix-turned-helix DNA binding motif, which binds to palindromic DNA sequences within promotor regions (Alekshun et al., 2001). The majority of MarR regulation involves export of the produced compounds, usually through drug efflux pumps, and therefore the molecules they regulate are usually exported out of the cell. This regulation of drug efflux pumps also makes the MarR family of interest when studying MDR bacteria (Blanco et al., 2016).

In some cases, antibiotics can act as regulators of secondary metabolism, with many intermediates or finished products binding to CSRs, both within the antibiotic's own BGC and disparate biosynthetic pathways. Jadomycin B biosynthesis in *S. venezulelae* is an example of one antibiotic regulating another. JadR1, the primary activator of the jadomycin BGC, represses chloramphenicol biosynthesis, although it is unclear if these interactions also involve the JadX regulator in the jadomycin BGC (Xu et al., 2010; Chater, 2016). The same jadomycin B BGC is also autoregulated by interactions between JadR1 and jadomycin B, with high concentrations of jadomycin B leading to dissociation of JadR1 from its target promotors, inhibiting jadomycin B biosynthesis (Kong et al., 2019).

1.4.2 Rare Actinomycetes

Actinomycetes whose isolation rate by conventional methods are significantly lower than that of *Streptomyces* species are known as 'rare actinomycetes.' These originate from various families within the actinobacteria class, including *Pseudonocardiaceae*, *Nocardiodaceae* and *Jiangellaceae*, and contain a vast previously untapped chemical diversity, including the potential to be a source of antibiotics. Examples of antimicrobial compounds originating from rare actinomycetes include; teicoplanin, used to treat MRSA and *Enterococcus faecalis,* isolated from *Actinoplanes teichomyceticu*s in 1978 and rosamicin, an antibiotic similar to erythromycin originating from *Micromonospora* in 1972. It is estimated that 16% of the total number of antibiotics originate from rare actinomycetes, mainly from the *Micromonosporaceae, Pseudonocardiaceae* and *Thermomonosporaceae* (Takahashi

and Nakashima, 2018). Many of these antibiotic-producing rare actinomycetes are found in soil but also in marine environments, deserts and freshwater ecosystems, that were underexplored during the golden age of antibiotics due to the perception that these niches would present overwhelming challenges for microbial life (van der Meij et al., 2017). Metagenomic studies of the 16S rRNA content of environmental soil samples have shown that only about 1% of bacterial life is culturable under standard laboratory conditions. With the advent of the iChip, isolation and culture of a more extensive selection of soil-dwelling bacteria, potentially including rare actinomycetes, is possible as the device allows for signalling molecules and growth components from the surrounding environment to enter the growth chambers (Nichols et al., 2010). The regulatory mechanisms of the rare actinomycetes are under-studied, and the few that have been studied have focussed upon the core regulatory genes discussed above, such as the *bld* family or upon areas where significant differences to *Streptomyces* species are expected. For example, Actinoplane sporulation produces zoospores with flagella designed to swim through aquatic environments to discover more amenable conditions for growth (Mouri et al., 2017; Mitsuyama, Tezuka and Ohnishi, 2019). This lack of understanding can complicate natural product discovery from these rare actinomycetes as the few regulatory pathways that have been studied have shown differences from those of *Streptomyces* species. For example, the regulation of an antifungal nystatin-like *Pseudonocardia* polyene (NPP) in *Pseudonocardia autotrophica* depends upon six pathway-specific regulators, unlike the one typically observed in *Streptomyces* biosynthetic pathways (Jeon et al., 2011).

1.5 Natural Product Discovery

Historically, most natural antibiotics were found using 'The Waksman Platform' where natural products extracted from bacteria or fungi were screened for their ability to inhibit culture-grown bacteria (Lewis, 2012). This was the approach used through most of the golden age of antibiotic discovery with great success, as illustrated in Figure 6. Potential antibiotics had to demonstrate broad-spectrum activity and minimal human toxicity to be usable in the clinic due to the inability to accurately identify the infecting organism. However, this has rediscovery issues, as

most natural products were isolated from soil-dwelling actinomycetes and many chemicals are shared across multiple genera in part due to *Streptomyces*' ability to share genetic material (Chater and Chandra, 2006; Wright, 2017). Recently, antibiotic discovery from actinomycetes has focused on three main areas; manipulating antibiotic production by utilising pleiotropic techniques, using genetic modification to mine the genome for cryptic BGCs and isolating actinomycetes from underexplored environments (Devine, Hutchings and Holmes, 2017).

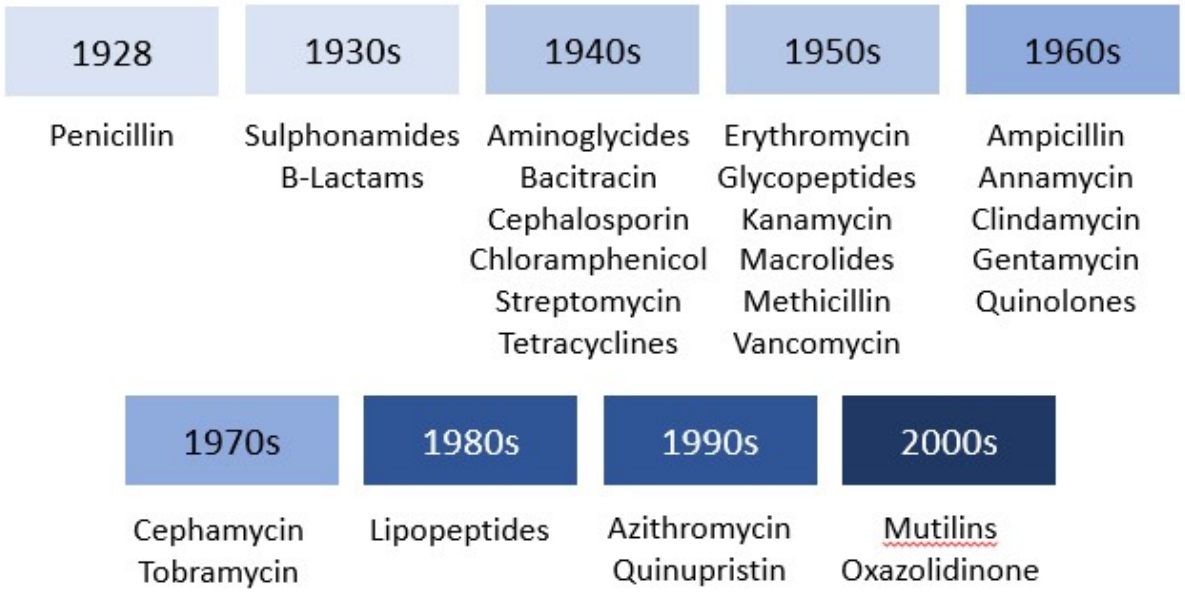


Figure 6 - The Golden Age of Antibiotic Discovery showing the decade various classes of antibiotics were discovered. Adapted from (Bbosa *et al.*, 2014). The majority of antibiotics were discovered in the 1940s-60s, with relatively few discovered since even as resistance to discovered antibiotics became more widespread.

1.5.1 Inducing Secondary Metabolite Biosynthesis

Secondary metabolite biosynthesis is under tight regulatory control in actinomycetes, affected by nutrient levels and signalling molecules from other organisms in competition in the surrounding environment (van der Meij et al., 2017). Therefore, a relatively simple way to alter secondary metabolite biosynthesis in actinomycetes is to manipulate the growth conditions in the laboratory to mimic environmental pressures by utilising different media compositions or using additives that mimic signalling molecules. These are known as pleiotropic methods. One such media supplement is N-acetylglucosamine (GluNAc), a carbon and nitrogen source

for *Streptomyces* species. It forms part of the bacterial peptidoglycan wall. GluNAc is a monomer of chitin, which forms fungal cell walls and inhibits *S. coelicolor* from progressing to sporulation when added to nutrient-rich media by preventing the global regulator DasR, which activates several genes involved in sporulation, from binding to its gene targets. This is in contrast to glutamate, another carbon and nitrogen source that has no impact on development (Van Wezel et al., 2005; Rigali et al., 2006). This leads to a reduced level of antibiotic production by most *Streptomyces* species when grown on media supplemented by GlcNac. However, under nutrient-poor conditions, GlcNAc supplementation increased antibiotic production. This is speculated to be due to GlcNAc accumulating during cell-wall hydrolysis, which occurs when nutrients are scarce, signalling that sporulation should begin (Rigali et al., 2008). An alternative media supplement is sodium butyrate. Sodium butyrate is a foul-smelling four-carbon fatty acid produced by bacteria, including some *Clostridium* species, and during human metabolism of dietary fibre (Seedorf et al., 2008; Morrison and Preston, 2016). Sodium butyrate has been studied primarily as a regulator in mammalian cells, where it is found to cause hyperacetylation of histones by inhibiting histone deacetylase, leading to various effects, such as inhibition of cell differentiation and inducing hormone production, depending on the cell affected (Kruh, 1981). Histone-like proteins have been found in various bacteria, including *S. coelicolor*, to act in a regulatory manner by altering DNA supercoiling and therefore access to genes by DNA-binding proteins (Aldridge et al., 2013). Various *Streptomyces* species contain histone deacetylases and histone deacetylase inhibitors. For example, Trischostatin A, originally used as an antifungal agent produced by *S. hygroscopicus*, was later found to have an inhibiting effect on histone deacetylases and was investigated as a potential anti-cancer agent (Yoshida et al., 2005; Ejje, Lacey and Codd, 2012). Marine *Streptomyces* species are particularly prolific producers of histone deacetylases, which could potentially be targeted by sodium butyrate and lead to a change in secondary metabolite biosynthesis (Varghese, Jayasri and Suthindhiran, 2015; Abdelfattah et al., 2018). Antibiotic synthesis is also affected by, and therefore can be influenced by, the availability of nutrients such as carbon, nitrogen and metals as well as environmental factors such as pH. *Streptomyces* species are able to selectivity prioritise some carbon sources

over others by carbon catabolite repression (CCR). Glucose is a high-quality carbon source for most bacteria in respects of cell growth and can deactivate elements of primary carbon metabolism, such as xylanase, chitinase, cellulase, β-galactosidases and proteolytic enzymes (Romero-Rodríguez et al., 2017). However, this repression effect by glucose limits its ability to stimulate antibiotic production as it represses gene expression of many antibiotics, including polyketides such as actinorhodin in *S. lividans* by repressing the transcription of the global regulator AfsR2 (Kim et al., 2001; Romero-Rodríguez et al., 2016). Other, less accessible carbon sources such as sucrose, glycerol and maltose have also been shown to influence antibiotic production. For example, cephamycin C synthetase in *S. clavuligerus* is suppressed by glycerol though not by other, more accessible forms of carbon like glucose (Lebrihi, Lefebvre and Germain, 1988). Interestingly, biosynthesis of the β-lactamase inhibitor clavulanic acid by *S. clavuligerus* is increased by glycerol even as cephamycin C production is suppressed (Sánchez et al., 2010). The full details of the mechanism of CCR in actinomycetes are not understood. It is known that there are several pathways, which vary for the exact species and carbon source combination being investigated. Therefore, it is of potential value to attempt multiple carbon sources when attempting to stimulate secondary metabolite production (Romero-Rodríguez et al., 2017). Similarly to carbon metabolism, nitrogen sources are also hierarchised by bacteria, affecting the secondary metabolic pathways that require nitrogen precursors, such as in the case of *S. fradiae* biosynthesis of tylosin, which is inhibited by ammonium (ŌMura et al., 1984). Additionally, *S. coelicolor* has been shown to grow in high concentrations of polyamines, such as putrescine and cadaverine, as the sole nitrogen source (Krysenko et al., 2017). Polyamines are toxic to most prokaryotic organisms, and their production is under strict regulatory control. Therefore this may mean that high levels of polyamines could repress antibiotic production (Romero-Rodríguez et al., 2018). Trace metal (Fe, Co, Cu, Mn, Ni, Zn and V) availability on actinomycete secondary metabolite production has also been shown to impact secondary metabolite biosynthesis. When grown using an iron-poor medium, *S. coelicolor* increased actinorhodin production, which was further increased when the nitrogen source was changed from ammonium to nitrate (Coisne, Béchet and Blondeau, 1999). This is regulated by IdeR, a DtxR family regulator that binds to 14

iron-metabolism-related genes in addition to several smBGCs by binding to palindromic sequences in the promotor regions of the BGC (Cheng et al., 2018). Other examples of trace metals effecting biosynthesis of antibiotics include nickel, cobalt and zinc inhibiting actinorhodin production in *S. coelicolor* and an increase in nosiheptide, a thiopeptide antibiotic, biosynthesis in *S. actuosus* Z-10 and geosmin in *S. halstedii* in the presence of zinc (Locatelli, Goo and Ulanova, 2016). Some antibiotics are affected by multiple nutrient stress conditions. For example, biosynthesis of the macrolide-class antibiotic AK-111-81 by *S. hygroscopicus* is influenced by; Mn2+, Cu2+, Fe2+ concentration, all of which stimulated AK-111-81 biosynthesis as metal concentration increased, the alteration of nitrogen source between a variety of ammonium salts, with ammonium succinate producing the highest rate of antibiotic biosynthesis, and the replacement of glucose with other carbon sources, such as lactose which reduced antibiotic production and fructose which increased it (Gesheva, Ivanova and Gesheva, 2005). This demonstrates how the effect various nutrient stresses described here have on secondary metabolite production cannot be taken purely in isolation, they are interconnected and changing multiple conditions simultaneously may induce the metabolism of novel antibiotics.

Osmotic stress has been demonstrated to impact the onset of aerial hyphae formation and sporulation (Bibb, 2005). Stress proteins control the response to osmotic pressure, including the response regulator OsaB, which is also observed in the transition between mycelium growth and aerial hyphae formation, immediately before secondary metabolite biosynthesis commences (Thomas et al., 2012). *S. coelicolor* mutants lacking OsaB or OsaC function are unable to erect aerial hyphae but produce five-fold greater quantities of actinorhodin and undecylprodigiosin than the wild-type strain (Bishop et al., 2004). Similar results were found in the commercially-used *S. avermitilis*, which produced three times greater quantities of the pesticide avermectin when OsaB was disrupted (Godinez et al., 2015). Therefore, it follows that by inducing osmotic stress by cultivating actinomycetes on media containing high concentrations of salts or sugars, it may be possible to activate otherwise cryptic BGCs encoding for novel antibiotics.

1.5.2 Genome Editing as a Method of Natural Product Discovery

Historically, pleiotropic methods have proven fruitful in antibiotic discovery. However, multiple secondary metabolites can be induced by one change, making identification and isolation of the antibiotic challenging. Ideally, individual BGCs would be activated in a controlled manner to allow for careful observation of the role of a particular secondary metabolite. Advancement in DNA sequencing techniques has allowed for the prediction of BGCs by analysing the modules present to compare them to known BGCs, using tools like antiSMASH (to reduce rediscovery) or to predict the chemical structure of the encoded compound (Blin et al., 2019). This ability to predict the structure of secondary metabolites from the BGC sequence is limited. Firstly due to errors that can be found even in high-quality genome sequences due to the repetitive nature of smBGCs, and secondly, due to the difficulty in determining the structure of non-iterative metabolic pathways (Rutledge and Challis, 2015). Thus, isolating the molecule and analysing it using traditional analytical chemistry techniques remains the optimal methodology to determine the structure of a secondary metabolite. Due to the genes encoding for the synthesis for a secondary metabolite usually being clustered with regulatory and export genes, heterologous expression of BGCs, where a BGC is inserted into a genetically amenable host, is possible. This allows for the production of the secondary metabolite from an isolated organism where little is known about ideal culture conditions or a gene identified from a metagenomic analysis, as well as unlocking cryptic BGCs that have resisted activation from pleiotropic techniques. Heterologous expression also allows genetic modification of the pathway to produce structural analogues or increase yields (Huo et al., 2019). *E. coli* is commonly used as a host organism for heterologous expression due to its fast growth, well-studied metabolic pathways and relative ease of modification. However, *E. coli* lacks phosphopantetheinyl transferase and some PKS precursors, such as methyl malonyl-CoA, meaning extensive genetic modification is often needed before the production of actinomycete-derived secondary metabolites in this host. To overcome this, *S. coelicolor* has been widely used as a heterologous host, often with native BGCs removed to allow carbon and nitrogen resources to be diverted toward the BGC of interest and to reduce the likelihood of a native antibiotic

obscuring results (Nepal and Wang, 2019). A 106kb BGC from *S. albus* DSM41398 encoding for salinomycin, an anticancer agent and veterinary antibiotic used in poultry production, has been heterologously expressed in *S. coelicolor* using linear plus linear homologous recombination-mediated recombineering (LLHR (J. Yin et al., 2015)). LLHR is mediated by the exonuclease RecE, a single-strand DNA binding protein RecT and the RecA repair protein and allows for recombination without the need for DNA replication and only minimal homology (30bp) between the host DNA and DNA being inserted (Wang et al., 2006; Fu et al., 2012). The 141kb BGC for vancoremysin, an antibiotic that shows activity against MRSA and VRE, originating from *Amycolatopsis sp.* DEM30355 has also been heterologously expressed in *S. coelicolor*. This was the first antibiotic originating from a rare actinomycete from the *Amycolatopsis* genus to be heterologously expressed (Kepplinger et al., 2018). In addition to transplanting a gene from an organism isolated in the laboratory, it is possible to heterologously express genes from environmental metagenomic samples by assembling the gene artificially and inserting it into a host organism. This allows access to genes from the 99% of bacteria unculturable under standard conditions (Nichols et al., 2010). There are two approaches to identifying molecules from metabolic libraries; functional screening and homology screen. Functional screening relies on an unbiased screen of individual clones, each containing a singular BGC encoding for a secondary metabolite from the metagenomic library, which is screened for bioactivity. Homology screening, meanwhile, relies on using the DNA sequence of known secondary metabolites to prioritise similar BGCs (Katz, Hover and Brady, 2016). Both of these culture-independent methods have drawbacks. Functional screening may take more time and resources as it analyses genes randomly. However, homology screening is less likely to find an antibiotic with a novel mechanism of action because it targets smBGCs similar to those already known to produce antibiotics. Additionally, both methodologies suffer from the low yields often associated with heterologous expression, and the inability for cross-cluster regulation to occur as only one BGC is present in each clone. As many BGCs require others for precursors or as regulators, this limits the success of these methods (Gomez-Escribano and Bibb, 2011; Katz, Hover and Brady, 2016). Due to the problems with heterologous expression, it is often preferable to express the BGC in

the native organism where it can be cultured meaning modification of the native organism may be required to unlock cryptic smBGCs. A variety of techniques can be used to genetically modify native producers, and one such example is ribosomal engineering. When mutations in the *rpsL* gene encoding ribosomal protein S12 were induced by culturing in the presence of low concentrations of streptomycin or tetracycline, *S. lividans* began to synthesis the otherwise cryptic antibiotic actinorhodin, likely by alterations to the translational machinery (Shima et al., 1996). Other studies using streptomycin, gentamycin and kanamycin have triggered point mutations in the *str* or *rpoB* genes, by causing these enzymes to be overproduced (Ochi et al., 2004). However, ribosomal engineering is still untargeted in that it may have no impact in some strains or may not activate the cryptic BGCs of interest. It is often better to mutate the BGC of interest, or regulators of it, in order to stimulate production of the secondary metabolite it encodes.

Genome editing in *Streptomyces* species has historically used homologous recombination, which is time and labour intensive compared to other species, often taking more than a month to modify a single gene. Typically, a single-crossover integration of a suicide plasmid was employed, disrupting the target gene. This process is limited by the need for selective markers within the gene of interest and the relative ease of the wild type being restored without the presence of selective pressure, such as an antibiotic for which the modified gene encodes resistance (Cobb, Wang and Zhao, 2015; Tong, Weber and Lee, 2019). This was later replaced by PCR targeting, allowing for larger deletions with reduced risks of frame-shift mutations compared to suicide plasmids. PCR targeting first uses recombination between a PCR-generated selectable marker, flanked by a short (<50nt) nucleotide sequence homologous to the desired region of the chromosome, and an antibiotic-resistance cassette in *E. coli.* The gene disruption cassette aac IV is then added to extracted plasmid using PCR. This can then be transformed into the *Streptomyces* strain of interest, which causes disruption of the targeted gene and then homologous recombination using the repair template provided (Gust et al., 2003). To increase efficiency, I-SceI endonuclease methods were later developed. These use enzymes from the meganuclease family I-SceI from *Saccharomyces cerevisiae* to cause a

double-strand break (DSB) at the targeted loci. Targeting is achieved by inserting an 18nt recognition sequence. The DBS leads to higher efficiency than previous methods as this style of break is more likely to trigger homologous recombination than single-strand breaks. I-SceI endonuclease methods allowed for further study for *Streptomyces* species, especially their DNA repair mechanisms, and reduced off-target mutations compared to both suicide plasmids and PCR targeting techniques (Siegl et al., 2010).

More recently, a gene editing technique using the high efficiency clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) mediated genome editing system has been developed for many prokaryotic and eukaryotic organisms. CRISPR/Cas9 is found in around 40% of bacteria and defends the cell against bacteriophage in the event of reinfection by a previously encountered phage, similar to acquired immunity in mammals (Mojica et al., 2000; Hsu, Lander and Zhang, 2014). The CRISPR array stores fragments of genetic material from a bacteriophage that has infected the cell but was resisted by it. These form the spacers in the array between the palindromic repeats. The CRISPR array is transcribed into single-stranded RNA before being processed into shorter CRISPR RNA (crRNA), which combines with trans-activating RNA (tracrRNA) and a Cas enzyme, such as the nuclease Cas9. This complex then patrols the cell until it encounters DNA that complements the viral genetic material encoded by the crRNA, which it binds to and cleaves to form a DSB. The cleavage also requires an NGG sequence, called the protospacer adjacent motif (PAM) sequence, to be present at the 3' end of the intended cleavage target. The DSB occurs at position three upstream of the PAM (Gupta and Musunuru, 2014; Hsu, Lander and Zhang, 2014). In 2015, Cobb and colleagues developed pCRISPomyces, an engineered plasmid containing all the genetic information for creating DSBs at a defined locus on the *Streptomyces* genomes. On the plasmid are genes encoding for Cas9 and a single guide RNA (sgRNA), which combines the roles of crRNA and tracrRNA to simplify the system and increase efficiency. The plasmid also contains the information required for the DSB to be repaired by homologous recombination, which allows for better control of the DNA repair process than non-homologous end joining that may otherwise occur

(Cobb, Wang and Zhao, 2015). This ability to have fine control over where genetic edits occur, combined with the relatively high yields, allows for deletion or disruption of pathways competing with cryptic smBGC activation to encourage the activation of these cryptic BGCs.

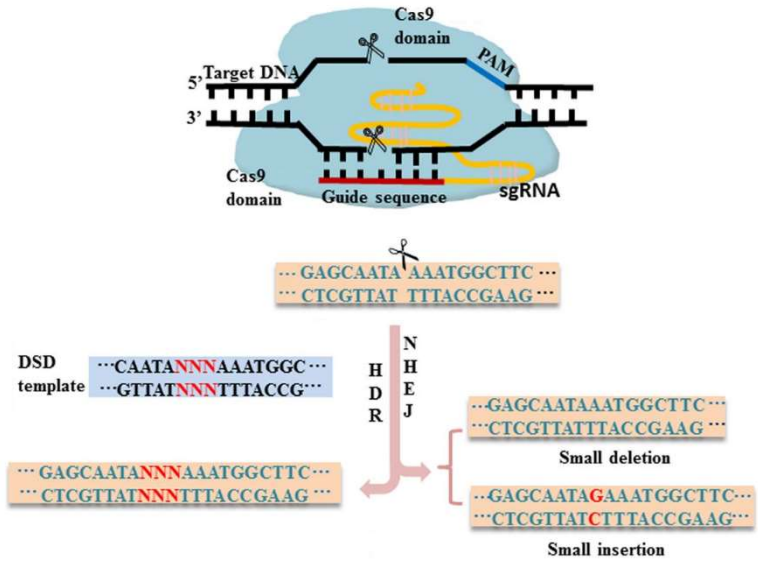


Figure 7 - The *pCRISPomyces* system, developed by Cobb *et al.* The synthetic guide RNA (sgRNA) confers target specificity and directs the location of the double strand break by Cas9 nuclease. A double strand DNA (DSD) template can then be used to direct DNA repair to insert or delete a region of DNA. Image adapted from (Xiao-Jie *et al.*, 2015).

By deleting gene clusters that utilise high quantities of carbon or nitrogen, other smBGCs may be activated as there are now sufficient resources for their secondary metabolites to be produced (Nepal and Wang, 2019). Alternatively, a deleted gene could encode a repressor of the cryptic BGC, as in the example of antimycin production by *S. albus* S4, which is regulated by FscRI, the ligand for which is produced by a disparate candicidin BGC (McLean, Hoskisson and Seipke, 2016). Another method is to mutant the CSRs in the BGC of interest to alter the expression of the secondary metabolite they produce by overexpressing activator genes or deleting repressor genes. One example in *S. coelicolor* demonstrated that production rates increased when CRISPR/cas9 was used to delete the *actORF1* and *actVB* genes responsible for regulating actinorhodin biosynthesis (Tong et al., 2015). More interesting in terms of novel antibiotic discovery is when LAL proteins were overexpressed in *S. ambofaciens* ATCC23877. The overexpression of the LAL proteins resulted in a previously-repressed glycosylated macrolide, stambomycin, to be

produced by a T1PKS. LAL proteins regulate many other cryptic genes in actinomycetes, so this method may lead to further antibiotic discovery (Laureti et al., 2011). CRISPR/Cas9 also allows for the rearrangement, deletion or addition of enzymatic domains within NRPS, PKS or RiPP synthesis pathways to alter the products these pathways create. This allows structural analogues of existing compounds to be designed and created as in the example of rapamycin analogues being produced following the modification of a PKS BGC in *S. rapamycinicus* (Wlodek et al., 2017). Software such as ClusterCAD can aid the design of these semi-synthetic PKS BGCs using rational design. Although this may limit the chances of antibiotics with novel mechanisms of action being produced, there is a possibility for resistance-breaking modifications to be discovered and incorporated into existing antibiotics or the improvement of other pharmacokinetic and pharmacodynamic properties of the molecule (Eng et al., 2018). Providing a high-quality genome sequence is available, CRISPR/Cas9, and more specifically the p-CRISPomyces system, can be a powerful method to exploit the otherwise cryptic BGCs in *Streptomyces* and rare actinomycetes isolated from the environment, potentially leading to the discovery of novel antibiotics.

1.5.3 Novel Environments as a Source of New Actinomycete Strains

The majority of actinomycetes that have been isolated have come from soil despite only 1% of soil bacteria being cultured. As previously discussed, the iChip is one method of increasing the variety of species of bacteria that can be isolated from the soil, which may include rare actinomycetes that are difficult to culture under standard laboratory conditions (Nichols et al., 2010). In addition to attempts to isolate more strains from soil environments, exploration of novel environments is ongoing. Actinomycetes have been isolated from marine sponges (Subramani and Sipkema, 2019), ice core samples in Tibetan glaciers (Shen et al., 2018), cave systems in Slovenia (Pašić et al., 2010) and highly-radioactive hydrothermal springs in Hungary (Enyedi et al., 2019). Additionally, actinomycetes have been found to form symbiotic relationships with many insect systems, including solitary digger wasps (Kaltenpoth, 2016), mosquitoes (Onchuru et al., 2016) and with fungus-growing ants (Currie et al., 2003). Recently the plant ant-associated *S. formicae* has yielded

formicamycin, an antibiotic with a novel mechanism of action, discovered by using the genome-mining techniques described above (Qin *et al.*, 2017a). This demonstrates that exploring these niches to discover new strains of actinomycetes and employing the techniques described previously to mine their genomes could prove fruitful in the search for novel antibiotics.

Alternatively, new methods have developed the possibility of culturing difficult to isolate actinomycetes from previously studied environments. Droplet microfluidics generates droplets of fluids with diameters in the femtometres scale, allowing individual cells to be separated from a bulk liquid. Within these droplets, cells are undisrupted by competitors and predators, allowing for the isolation of bacterium that may otherwise not survive classical cultivation methods (Huys and Raes, 2018). Further, studies involving *Bacillus pumilus* were able to co-culture two different strains together in adjacent droplets, where an otherwise cryptic antibiotic encoded in the *B. pumilus* genome was activated to inhibit a strain of *S. aureus* isolated from the oral cavity of a Siberian brown bear (Terekhov *et al.*, 2018) If similar techniques could be applied to actinomycetes, this may unlock chemical diversity in both newly and previously-isolated strains. In addition to potentially allowing for the cultivation of novel actinomycetes, being contained within a droplet prevents diffusion of signalling molecules away from the cell, potentially simulating high cell density environments despite actually being isolated. This may allow signalling molecules to accumulate to levels that influence secondary metabolism, activating otherwise cryptic secondary metabolomic pathways (Van Den Berg *et al.*, 2010).

1.6 Ant-Actinomycete Mutualism as a Source of New Actinomycete Strains

Fungal agriculture amongst ant species has evolved twice – once in the attine ants during the Eocene climatic optimum, approximately 50 million years ago in the Neotropics, and the second time by some species within the *Megalomyrmex* that exist as a trophic parasite of attine ant gardens, occasionally turning aggressive and usurping them. Today there are over 230 extant species, with another 21 known to be extinct (Bolton, no date; Mueller et al., 2001; Schultz and Brady, 2008). These fungus growers are dependent upon their fungal cultivar. When a daughter queen

leaves a colony to mate and establish a new colony, she carries a nucleus of the fungus, which can nucleate the new fungal garden. This makes fungus-growing ants especially vulnerable to opportunistic pathogens, which can target either the ants or their budding cultivar, and predation during the initial colony founding stage (Fernández-Marín, Zimmerman and Wcislo, 2004). There are five biologically distinct agricultural systems within the attine ants that are practised in extant species; lower attine agriculture, coral fungus agriculture, yeast agriculture, higher attine agriculture and leaf-cutter agriculture. The lower attine agriculture system most closely mirrors the original system that evolved 50 million years ago (Schultz and Brady, 2008). Lower attine fungal cultivars consist of a fungus from a single paraphyletic grade of *Leucocoprineae* and are fully capable of existing free of the attine symbiotic relationship, showing minimal adaptations since domestication (Vo, Mueller and Mikheyev, 2009). Yeast agriculture also utilises *Leucocoprineae* for their fungal cultivar but has co-evolved over an extensive period, resulting in a unique phylogeny. Yeast agriculture-based cultivars consist of clusters of single-celled yeast phase fungal growth instead of the mycelium growth observed in all other attine agricultural systems, although this has not prevented the *Leucocoprineae* from being able to exist separate from the ant cultivar (Mueller, Rehner and Schultz, 1998; Schultz and Brady, 2008). Ants in lower and yeast-agricultural systems collect fallen flowers, dry plant debris (such as wood), seeds and insect frass upon which their fungal cultivar is propagated (De Fine Licht and Boomsma, 2010).

Coral fungus agriculture is the only known transition away from a *Leucocoprineae* cultivar, instead cultivating *Pterulaceae*. *Pterulaceae*-based cultivars are infected by a specialised clade of *Escovopsis*, derived from *Escovopsis* infecting the lower attine agricultural systems. Some members of this clade then went on to evolve the ability to infect the cultivars of higher attine agricultural systems (Gerardo, Mueller and Currie, 2006). Ants utilising a coral agricultural system collect similar substrates for their fungal cultivars as the lower- and yeast-agriculture systems. However, some species also collect nectar and insect carcasses (De Fine Licht and Boomsma, 2010). Leafcutter agriculture is a sub-division of higher attine agriculture, having evolved 8-12 million years ago. Both utilise cultivars evolved from the lower attine agriculture

cultivars. However, the higher attine agriculture cultivars are far more domesticated, being unable to survive independently of the ants and producing hyphal tips, called gongylidia. These gongylidia contain high concentrations of lipids and carbohydrates for the ants alongside fungal spores and digestive enzymes, such as pectinases and proteinases, which the ants excrete in their faeces. The ants spread their faeces on newly-delivered plant material, which has been chewed by the ants, depositing the fungal enzymes and spores to propagate their fungal cultivar (De Fine Licht, Boomsma and Tunlid, 2014). Leafcutter agriculture, consisting of the *Atta* and *Acromyrmex* genera, generally has higher yields of gongylidia than their non-leafcutting higher agriculture counterparts, partly due to the improved ability of their fungal cultivars to process chitin (Nygaard et al., 2016). Higher attines use the same substrates as other agriculture systems except for collecting live plant material. This is especially noticeable in the leafcutter ants, where the majority of the fungal cultivar is grown upon freshly-cut plant material (De Fine Licht and Boomsma, 2010).

All the agriculture systems cultivate fungal gardens in a symbiotic relationship between the ants, their fungal cultivar, and actinomycetes, such as *Streptomyces* and *Pseudonocardia* species, which assist in the protection of the fungal cultivar from the parasitic *Escovopsis* fungus (Schultz and Brady, 2008). *Escovopsis* is a specialist pathogenic fungus found in approximately a quarter of all fungus-grown ant colonies but has not been found in the wider environment (Gerardo et al., 2004). *Escovopsis* is transmitted between colonies, and most *Escovopsis* species within a paraphyletic grade target one of the different agricultural systems and rarely transmit across agricultural systems, although some generalist species, such as *E. trichodermoides,* have been shown to produce chemicals that interfere with many different fungal cultivars (Currie, Mueller and Malloch, 1999; Schultz and Brady, 2008; Bizarria, Nagamoto and Rodrigues, 2020). As the fungal cultivar is the exclusive nutrient source for the queen and larvae ants, if *Escovopsis* overwhelms a fungal cultivar, the associated ant colony will eventually collapse. To defend from *Escovopsis,* the ants have evolved specialised behaviours to detect the pathogen's presence and remove it from the nest, taking it to distant waste piles after rubbing the infected garden fragment on their carapace. Upon their carapace, fungus-growing ants maintain

biofilms of actinomycetes, most notably *Streptomyces* and the rare actinomycete *Pseudonocardia*. These actinomycetes are maintained in crypts in the ant carapace, with nutrients provided by the ant via exocrine glands (Currie et al., 2006). The lower-agricultural *Mycocepurus* possess these actinomycete biofilms-filled crypts on both their chest plate and under their forelegs. However, most ant species have crypts in only one of these locations. The leaf-cutting *Acromyrmex*¸ have white biofilms visible to the eye on their chest plates whilst the coral-fungus growers *Apterostigma* and lower-agricultural *Myrmicocrypta* maintain their actinomycete biofilms under the forelegs. These actinomycetes produce antibiotics and antifungal agents, which assist in suppressing the levels of *Escovopsis* in the cultivar and inhibiting the growth of those fragments of infected cultivar that are spread across the ant carapace. They are vertically transmitted both within colonies by mature ants rubbing newly-hatched larvae on their carapace to transfer the bacteria present and across colonies by the daughter queen carrying a sample of the actinomycete when establishing the new colony (Currie et al., 1999). Once established, the colony then goes on to recruit new actinomycetes by sampling from the environment. This allows for a diverse range of antifungal and antibiotic compounds to be present in the colony, decreasing the ability for *Escovopsis* to develop resistance as it must overcome multiple antifungal agents simultaneously (Holmes et al., 2016).

Pseudonocardia is a rare actinomycete that is commonly isolated from leaf-cutter ant colonies, where they produce antifungals to help protect the colony from *Escovopsis*. *Pseudonocardia* strains isolated from the lower attine *Apterostigma dentigerum* produce dentigerumycin, and *Pseudonocardia* strains isolated from the colony of the higher attine *Trachymyrmex cornetzi* produce gerymycins. Both of these compounds are cyclic depsipeptides capable of inhibiting the growth of *Escovopsis* (Oh et al., 2009; Sit et al., 2015). Additionally, a *Pseudonocardia* strain associated with leafcutters *Acromyrmex octospinosus* was found to produce the polyene antifungal nystatin P1 (Barke et al., 2010). Ant-associated *Pseudonocardia* species are divided into *P. octospinosus* – the Ps1 phenotype, and *P. echinatior* – the Ps2 phylotype. An individual colony rarely contains both phylotypes, even where multiple queens cofound a single colony (Poulsen et al., 2005). These two phylotypes encode for

multiple BGCs not present in the other phylotype, with few BGCs shared between the two. All strains from both phylotypes encode for a nystatin-like antifungal, with Ps1 strains encoding nystatin P1 and Ps2 strains encoding unidentified novel nystatin (Holmes et al., 2016).

1.7 20 Antibiotic-Producing Actinobacteria Isolated from Fungus-Growing Ant Nests

High-quality genome sequencing of 20 actinomycete strains isolated from fungus-growing ant nests has been performed, Section 2.2. An initial assessment of their BGC content and phylogeny was performed by Dr Neil Holmes (former post-doctoral researcher, Hutchings Laboratory). This showed that collectively the strains possessed 394 collective BGCs, of which 75 were RiPPs and 63 were NRPS BGCs, with 93.3% of the BGCs unique to a single strain. Predictions of the bioactivity of the strains using antiSMASH 4.0 revealed that several strains encoded antifungal or antibacterial agents (Blin et al., 2017). *Streptomyces* KY1 encodes candicidin and antimycin, whilst *Streptomyces* KY2 contains a BGC encoding for a candicidin-like polyene. *Pseudonocardia* P1 and *Pseudonocardia* UM4 encode nystatin-like clusters similar to nystatin P1, whilst the other two *Pseudonocardia* strains, UM9 and UM14, do not encode polyene antifungals (Barke et al., 2010). This aligns with previous observations that lower attine-associated *Pseudonocardia* strains do not encode polyene antibiotics.

1.8 Thesis Objectives

This thesis investigates the antibiotic and antifungal production of 20 actinomycete strains isolated from fungus-growing ant colonies. The biological activity of 18 strains was determined across media containing a diverse range of substances including varied carbon and nitrogen sources. Their genomes and phylogenies were interrogated thoroughly to determine what smBGCs are present. For a select number of strains where biological activity could not be easily explained by smBGCs identified by antiSMASH analysis, attempts were made to isolate and identify the bioactive compound. Additionally, CRISPR/Cas9 was used on a selection of strains to remove

the strain's capability to synthesise bioactive molecules by targeting the smBGCs with high levels of homology with BGCs encoding for known antibiotics or antifungals.

2 Materials and Methods

2.1 Chemical Reagents

Chemicals and reagents used are laboratory grade or higher, purchased from Sigma Aldrich (UK) or Thermo Fisher Scientific (UK) unless otherwise stated. All media and solutions were produced using deionised water unless stated otherwise.

2.2 Bacterial Strains

The bacterial strains used in this study are described in

Table **1**. *B. subtilis, E. coli* and *C. albicans* strains were grown at 37°C either plated upon LB agar or whilst being shaken at 220rpm in LB broth. All Actinomycete strains were grown at 30°C and shaken at 220rpm upon the growth media listed in Table 2. Table 3 presents the antibiotics used in this work and the concentrations used to ensure selection.

Table 1 - List of Bacterial Strains Used in this Work

Strain	Description	Gen Bank Accession Number	Plasmid	Resistance	Source/Reference
B. subtilis					
168	ATTC 23857		-	-	Gift from Dr Neil Holmes
E. coli					
NCTC 12923	NCTC 12923		-	-	Gift from Dr Neil Holmes
KanR12923	NCTC12923ΔpET28a		pET28a	KanR	This work

C. albicans					
ATCC MYA 2876	ATCC MYA 2876		-	-	Gift from Dr Neil Holmes
Pseudonocardia					
UM4	Isolated from *Trachymyrmex turrifex* in Texas, USA	QJPN00000000	-	-	Gift from Ulrich Mueller, Texas
UM14	Isolated from *Cyphomyrmex wheeleri* in Austin, Texas, USA	CP029798	-	-	Gift from Ulrich Mueller, Texas
UM9	Isolated from *Mycocepurus smithii* in Pina, Panama	QKQY00000000	-	-	Gift from Ulrich Mueller, Texas
P1	Isolated from *Acromyrmex octospinosus* in Trinidad	ADUJ00000000	-	-	(Barke et al., 2010)
PS2	Isolated from *Acromyrmex echinatior* in Panama	QJPM00000000	-	-	This work
Ae707	Ps1-type *Pseudonocardia*		-	-	Gift from Dr Neil Holmes
Ae717	Ps2-type *Pseudonocardia*		-	-	Gift from Dr Neil Holmes
Streptomyces					

KY2	Isolated from *Tetraponera penzigi* in Kenya	CP029636			(Seipke et al., 2013)
B2	Isolated from *Acromyrmex echinatior* in Panama.	QHCI00000000			This work
FG4	Isolated from *Allomerus decemarticulatus* in French Guiana	QFZS00000000			(Seipke et al., 2013)
FG7	Isolated from *Allomerus decemarticulatus* in French Guiana.	CP029633			(Seipke et al., 2013)
A7	Isolated from *Acromyrmex echinatior* in Panama	CP029637			This work
KY4	Isolated from *Tetraponera penzigi* in Kenya	QHCH00000000			(Seipke et al., 2013)
FG1	Isolated from *Allomerus octoarticulatus* in French Guiana	QFZR00000000			(Seipke et al., 2013)
KY1	Isolated from *Tetraponera penzigi* in Kenya	QHCG00000000			(Seipke et al., 2013)
Amycolatopsis					

UM15	Isolated from *Mycocepurus smithii* in Iguazu, Argentina	QJPO00000000			(Seipke et al., 2012, 2013)
FG22	Isolated from *Allomerus decemarticulatus* in French Guiana	CP029634			(Seipke et al., 2013)
Other Actinomycetes					
Agrococcus A6	Isolated from *Acromyrmex echinatior* in Gamboa, Panama.	CP029799			This work
Tsukamurella FG11	Isolated from *Allomerus decemarticulatus* in French Guiana.	CP029635			(Seipke et al., 2013)
Tsukamurella E7	Isolated from *Acromyrmex octospinosus* in Trinidad	QIYX00000000			(Barke et al., 2010)
Nocardiopsis E5	Isolated from *Acromyrmex octospinosus* in Trinidad	QIYY00000000			(Barke et al., 2010)
Jiangella S1	Isolated from *Acromyrmex echinatior* in Panama	QIYZ00000000			This work

Table 2 - List of Media used in this Work

Medium	Recipe (per L)	Water Source	Source
LB	5g Yeast Extract, 10g NaCl, 10g Tryptone	dH_2O	(Bertani, 1951)
SFM	20g Soya Flour, 20g Mannitol, 20g Agar	Tap	(Kieser *et al.*, 2000)
SFMNAG	SFM with the addition of 2000µL filter sterilised 4g/80mL N-acetyl glucosamine after autoclaving	Tap	
SFMSB	SFM with the addition of 10mL filter sterilised 10M sodium butyrate after autoclaving	Tap	
YP	10g Yeast extract, 20g bacteriological peptone, 20g agarose	dH_2O	(Jones *et al.*, 2017)
YPD	YP with the addition of 100mL filter sterilised 20% glucose solution after autoclaving	dH_2O	(Jones *et al.*, 2017)
YEME	3g Yeast Extract, 5g Peptone, 3g Malt Extract, 10g Glucose, 20g agar	dH_2O	(Kieser *et al.*, 2000)
SPY	15g pancreatic digest of casein, 15g peptic digest of animal tissue, 2g Yeast Extract, 10g $MgSO_4$, 50mg $CaCl_2$, 30mg $ZnSO_4$, 25mg $FeSO_4$, 16mg $CuSO_4$, 12mg $MnSO_4$, 20g agar Adjust pH to 7.2 with NaOH Autoclave and then add 30g Soluble Starch before autoclaving again	dH_2O	(Imai *et al.*, 2015)

FML	10g Peptone, 20g Malt Extract, 40g Glucose, 20g Agar	dH_2O	
MYM	4g Maltose, 4g Yeast Extract, 10g Malt Extract, 20g Agar after autoclaving add 2mL Trace Element Mix[1]	50:50 Tap:dH_2O	(Stuttard, 1982)
IMA	50g Smash (Tesco, UK), 20g Agar	Tap	(Joshi *et al.*, 2010)
GYM	4g Glucose, 4g Yeast Extract, 10g Malt Extract, 2g $CaCl_2$, 15g agar	dH_2O	
Minimal	0.5g L-asparagine, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.01g $FeSO_4.7H_2O$, 10g agar, adjust pH to 7.2 with HCl Autoclave and then add 20mL of 50% W:W filter sterilised glucose	dH_2O	(Kieser *et al.*, 2000)
MinNAG	Minimal with the addition of 2000µL filter sterilised 4g/80mL N-acetyl glucosamine after autoclaving	dH_2O	
SM3	5g Glucose, 50g Maltodextrin, 25g Soya flour, 3g Beet Molasses, 0.25g K_2HPO_4, 2.5g $CaCO_3$, 20g Agar, adjust pH to 7.0 with KOH	dH_2O	This work
SM5	20g Peptone, 8g Lab Lemco (Oxoid, UK), 15g Glucose, 10mL 20% Glycerol solution, 0.4g $CaCO_3$, 20g Agar, adjust pH to 7.2 with KOH	dH_2O	This work
SM6	40g Corn Steep Liquor, 20g Maltodextrin, 2.5g NaCl, 0.5g $MgSO_4$, 20g Agar, adjust pH to 7.0 with KOH	Tap	This work

SM7	20.9g (3-(*N*-morpholino) propane sulfonic acid, 15g L-Proline, 20mL 20% Glycerol solution, 2.5g Sucrose, 1.5g Sodium L-Glutamate monohydrate, 0.5g NaCl, 2g K_2HPO_4, 10mL 0.2M $MgSO_4$, 10mL 0.02 M $CaCl_2$, 5mL Trace Element Mix[1], 20g Agar, adjust pH to 6.5 with KOH	dH_2O	This work
SM12	10g Soya Flour, 50g Glucose, 4g Peptone (Formedium, UK), 4g Beef Extract Power, 1g Yeast Extract (Merck, Germany), 2.5g NaCl, 5g $CaCO_3$, 20g Agar, adjust pH to 7.6 with KOH	Tap	This work
SM14	10g Glucose, 20g Soya Peptone, 5g Lab Lemco (Oxoid, UK), 5g NaCl, 0.01g $ZnSO_4 \cdot 7H_2O$, 20g Agar, adjust pH to 7.0 with KOH	dH_2O	This work
SM15	20.9g (3-(*N*-morpholino) propane sulfonic acid, 11.5g Casamino acids, 23g 20% Glycerol Solution, 0.5g NaCl, 0.52g K_2HPO_4, 0.25g EDTA, 0.49g $MgSO_4 \cdot 7H_2O$ 0.49 g, 0.029g $CaCl_2 \cdot 2H_2O$ 0.029 g, 5mL Trace Element Mix[1], 20g Agar, adjust pH to 6.5 with KOH	dH_2O	This work
SM18	15g Glucose, 40g Soluble Starch, 20g Beet Molasses, 8g $CaCO_3$, 20g Agar	Tap	This work

SM19	40g Tomato paste (Kyknos, Greece), 15g Oat flour (Avenaflo, UK), 2g Glucose, 20g Agar	Tap	This work
SM20	20g Maltose, 5g Peptone, 5g Lab Lemco (Oxoid, UK), 3g Yeast extract (Oxoid, UK), 3g NaCl, 1g $MgSO_4 \cdot 7H_2O$, 20g Agar	Tap	This work
SM25	10g Peptone, 21g Malt extract, 40g 20% Glycerol Solution, 20g Agar	dH_2O	This work
SM30	40g Tomato paste (Kyknos, Greece), 15g Oat flour (Avenaflo, UK), 2g Glucose, 20g Agar, adjust pH to 4.5 with HCl	Tap	This work
SM32	10g Peptone, 21g Malt extract, 40g 20% Glycerol Solution, 20g Agar, adjust pH to 4.5 with HCl	dH_2O	This work
GYM+10μg/mL streptomycin	GYM with the addition of 200μL 50mg/mL streptomycin		
GYM+2μg/mL streptomycin	GYM with the addition of 4μL 50mg/mL streptomycin		
YP/C	YP with the addition of 2g $CaCO_3$		
ISP2	4g Yeast Extract, 10g Malt Extract, 4g Dextrose, 20g Agar	dH_2O	(Shirling and Gottlieb, 1966)
ISP4	10g Soluble Starch, 1g K_2HPO_4, 1g $MgSO_4$, 1g NaCl, 2g $(NH_4)_2SO_4$, 2g $CaCO_3$, 1mg $FeSO_4$, 1mg Cl_2Mn, 1mg $ZnSO_4 \cdot 7H_2O$, 20g agar	dH_2O	(Shirling and Gottlieb, 1966)
SFMCasAA	SFM with the addition of 11.5g Casamino acids		

2YT	16g Tryptone, 10g Yeast Extract, 5g NaCl	dH_2O	(Kieser *et al.*, 2000)
DNA	23g Difo Nutrient Agar	dH_2O	(Kieser *et al.*, 2000)

1 – Trace element mix consists of the following per 1L: 0.04g · $ZnCl_2$, 0.2g $FeCl_3 \cdot 6H_2O$, 0.01g $CuCl_2 \cdot 2H_2O$, 0.01g $MnCl_2 \cdot 4H_2O$, 0.01g $Na_2B_4O_7 \cdot 10H_2O$, 0.01g $(NH_4)6Mo_7O_{24} \cdot 4H_2O$

Table 3 - Antibiotics and Their Selection Concentrations

Antibiotic	Selection concentration (µg/mL)
Ampicillin	100
Apramycin	50
Chloramphenicol	30
Erythromycin	10
Hygromycin	50
Kanamycin	50
Nalidixic Acid	25
Streptomycin	50

2.3 Glycerol Stocks of Strains

Glycerol stocks of *B. subtilis, E. coli* and *C. albicans* were produced by centrifuging an overnight culture in 10mL LB broth culture at 400rpm for 10 minutes and resuspending the produced pellet in 4mL of 20% glycerol in LB broth. These were then stored at -80°C.

For actinomycete stocks, a confluent lawn was grown from a single colony on SFM at 30°C for the time described in Section 3.1. 10mL of ice-cold dH_2O was placed onto the plate, and a cotton bud was used to scrub spores from the colony. The resulting solution was then centrifuged at 4°C for 10 minutes at 4000rpm, and the pellet was resuspended in 2mL of ice-cold 20% glycerol solution, which was then stored at -80°C.

2.4 Phylogenetic Analysis

Phylogenetics was performed first by extracting the 16S rRNA gene sequences from the full genomes using RNAmmer 1.2 (Lagesen *et al.*, 2007). A general time reversible model was selected to build the phylogenetic tree, with *B. subtilis* ATCC 23857 as an outgroup. *Streptomyces coelicolor* A3(2), *S. venezuelae_JMC_4526* and *S. griseus* KACC 20084 were also included as model organisms. Alignment was performed in SILVA, set to the SSU alignment setting, with the tree built in the Denovo workflow in the FastTree program with a bootstrap value of 1000 (Pruesse, Peplies and Glöckner, 2012). This output was then visualised using the Interactive Tree of Life 6.5.8 (Letunic and Bork, 2021).

2.5 Light & Scanning Electron Microscopy

Light Microscopy was used to check for sporulation of actinomycetes strains using a standard light microscope. Scanning electron microscopy (SEM) was performed on a GeminiSEM 360 FEG (Ziess, UK) equipped with a PP3010 Cyro Preparation System (Quorum, UK). To prepare SEM samples, actinomycetes were grown as a confluent lawn on SFM for the time specified in Section 3.1, and a minimum of three 1cm^2 sections were removed. Up to 6 sections were simultaneously loaded into the PP3010 Cyro Preparation System by plunging the samples into liquid nitrogen in a vacuum and then transferred to the cyro-preparation chamber. Sublimation was performed at -90°C for 4 minutes before sputtering was performed using platinum nebulised by argon gas with a 5mA current for 50 seconds. Once loaded into the microscope, images were taken at various magnifications and working distances, detailed in the individual images.

2.6 Solid culture Biological Assays of Actinomycetes

Bioassays were performed by spotting 1µL of actinomycete spore stock onto the centre of 60mm plates and incubated at 30°C for the number of days specified in Section 3.1. On this day, soft-LB medium – made by following the standard LB recipe described in section 2.2 but only including half the mass of agar, was inoculated with sub-cultured overnights of the indicator strain when said sub-cultures possessed an OD_{600} between 0.5 and 0.7 when measured using a standard spectrophotometer.

10mL of inoculated LB broth was used per 100mL of soft-LB. 1mL of the resulting inoculated soft-LB was pipetted onto the plate containing the mature actinomycete colony, ensuring the soft-LB covered the entirety of the plate and that the actinomycete was not growing upon. These plates were grown overnight at 30°C, and then the zone of inhibition was measured using a ruler. Negative controls of uninoculated media plates incubated alongside those inoculated with actinomycete being tested. In all cases, indicator strains grown on these negative controls successfully grew confluent lawns, so are not separately reported.

2.7 Liquid Culture Biological Assays of Actinomycetes

To cultivate the actinomycetes in liquid culture, 5µL of spore stock was added to 100mL of liquid medium in a 250mL conical flask, which had a coiled spring in to prevent the clumping of cells. These were then incubated at 30°C for 14 days at 200rpm. After this period, the flasks were decanted into two 50mL centrifuge tubes and centrifuged at 4000rpm for 10 minutes. The supernatant was decanted into glass tubes and dried using an EZ-2 Elite 3.0 Personal Evaporator (Genevac, UK) on its HPLC-lyo setting until the resulting powder was dry. This was then resuspended in the minimum amount of dH_2O required, using sonication where needed, and then 10µL was pipetted onto a plate containing soft-LB inoculated with the indicator strain in the same way described in section 2.6. Negative controls of uninoculated media were processed in the same manner and did not disrupt the indicator strain's growth in any condition.

2.8 DNA Extraction and Manipulation

Genomic DNA of *E. coli* and actinomycetes was extracted using a Wizard Genomic DNA Purification kit (Promega, US) following the manufacturer's instructions. Plasmid DNA was prepared using a QIAprep Spin Miniprep kit (QIAGEN, Germany) per the manufacturer's instructions. To perform restriction digestions, , restriction enzymes from New England Biolabs (US) were used to digest PCR fragments and plasmid DNA in 25µL volumes. Digestions were performed at 37°C for one hour before inactivating the enzymes at 65°C for 15 minutes, and then, to prevent re-ligation, 2µL shrimp alkaline phosphatase was added to dephosphorylate the

digested DNA. The resultant digests were then analysed by gel electrophoresis, as described in section **Error! Reference source not found.**.

Ligations utilised T4 DNA Ligase (New England Biolabs, US), as per manufacturers' instructions. Reactions were conducted at a 3:1 ratio of plasmid to insert, with volumes calculated using the NeBio Ligation Calculator (last accessed 12/07/2022, available at: https://nebiocalculator.neb.com).

Golden Gate assembly was conducted using 100ng of purified backbone and 0.3uL of insert were combined with 2µL T4 ligase buffer (NEB, US) and 1µL T4 ligase (NEB, US) with 1µL BbsI (NEB, US) and made up to 20µL with dH_2O. The constructs were then assembled in a thermocycler under the following conditions:

- Ten cycles of the following:
    - 10 minutes at 37°C
    - 10 minutes at 16°C
- 5 minutes at 50°C
- 20 minutes at 65°C
- 4°C hold

Assembly of 20bp or smaller inserts, such as synthetic protospacers, were confirmed by blue/white screening using X-Gal and sequencing by Eurofins. Larger inserts were confirmed using colony PCR, as per Section 2.10, or restriction digestion, described in Section **Error! Reference source not found.**, followed by gel electrophoresis, as per Section **Error! Reference source not found.**.

To perform a Gibson Assembly, DNA fragments were incubated at a ratio of 1:3 plasmid and insert in the presence of Gibson Assembly Master Mix (NEB, US) for 1 hour at 50°C. 2µL Shrimp alkaline phosphatase was added to dephosphorylate DNA and prevent unwanted enzymic activity.

Where genes were synthesised, this was performed by Genscript (US)

2.9 Transformation of Chemically Competent *E. coli*

25µL of *E. coli* dH5α was combined with 2µg of DNA, mixed gently with a pipette tip and incubated on ice for 30 minutes. The mixture was heat-shocked at 42°C for 30 seconds before being placed back into ice for 2 minutes. 975µL of LB broth was then added and incubated whilst shaking at 220rpm at 37°C for 1 hour. The cells were then plated on solid agar LB, containing appropriate antibiotics, overnight at 37°C.

2.10 Colony PCR of *E. coli*

Following overnight incubation, a pipette tip was used to pick single colonies, transferring them to a 0.2mL PCR tube and discarding the tip into 500µL LB broth. This broth was kept at 37°C whilst BioTAQ PCR was performed as per Section 2.13. The reactions were analysed by gel electrophoresis, described in Section **Error! Reference source not found.**, with positive colonies transferred to 10mL of LB broth, supplemented with appropriate antibiotics. These were then incubated overnight whilst shaking at 220rpm at 37°C.

2.11 Conjugation into Actinomycetes

Single colonies of non-methylating *E. coli* pUZ8002, containing the desired plasmid, were selected from plates and cultivated in 10mL LB broth, supplemented with appropriate antibiotics, overnight at 37°C overnight at 220rpm. These were then subcultured until said sub-cultures possessed an OD_{600} between 0.5 and 0.7 when measured using a standard spectrophotometer. These were then washed twice to remove antibiotics by centrifuging at 4000rpm for 10 minutes, pouring off the supernatant and resuspending the pellet in 10mL of LB. To encourage germination, 100µL of actinomycete spores were mixed with 500µL 2YT, heat shocked at 50°C for 10 minutes, and added to the washed *E. coli* cells. The mixture was centrifuged at 13000rpm for 2 minutes, the supernatant removed, and the cells resuspended in the residual liquid. These were then plated on SFM, supplemented with 10mM $MgCl_2$ at various dilutions and incubated at 30°C for 16-20 hours. For the selection of desired ex-conjugants, 0.5mg Nalidixic acid and an appropriate concentration of the selection antibiotic, to give the final concentrations described in Table 3, were mixed

in 1mL dH_2O, which was then added to each plate, and cultures returned to the 30°C incubators for five days or until colonies appeared.

2.12 Colony PCR of Actinomycetes

Single colonies of actinomycete ex-conjugants were picked and soaked in 100µl 50% DMSO at 50°C for 1 hour. This was then used as a template for BioTaq PCR, described in Section 2.13, at 10% of the final volume of the reaction.

2.13 Polymerase Chain Reaction (PCR) and Gel Electrophoresis

Primers were designed manually using A Plasmid Editor 3.1.1 (Davis and Jorgensen, 2022) and ordered from Integrated DNA Technologies (IDT, US).

TaqBIO DNA polymerase (PCR Biosystems, UK) was used for diagnostic PCR, whereas Q5 High-Fidelity DNA Polymerase (NEB, US) was used to amplify DNA fragments destined for use in cloning.

Table 4 - PCR Conditions

Cycle(s)	Temperature (°C)	Time	Step
1	95	2 min	Initial denaturation
30	95	30 sec	Denaturation
	55-72[1]	30 sec	Anneal
	72	30 sec per kb	Extension
1	72	10 min	Final extension
1	4	hold	Final hold

1 – Anneal temperatures were calculated using the NEB calculator at www.tmcalculator.neb.com

Table 5 - Reaction Mix for 20µL TaqBIO PCR

Reagent	Volume (µL)	Final concentration
2x PCRBIO Taq Mix	10	1x
DMSO	1	5%
Forward primer (5µM)	0.5	125nM
Reverse primer (5µM)	0.5	125nM

Template DNA	0.5	Variable[1]
dH_2O	Up to 7.5[2]	

1 – Up to 10ng of template plasmid or DNA was added

2 – dH_2O was added in sufficient quantities to make up the reaction volume

Table 6 - Reaction Mix for 50µL Q5 PCR

Reagent	Volume (µL)	Final concentration
5X Q5 Reaction Buffer	10	1x
5X Q5 High GC Enhancer	10	1x
10mM dNTPs	1	200µM
Forward primer (10µM)	0.5	100nM
Reverse primer (10µM)	0.5	100nM
Template DNA	0.5	Variable[1]
Q5 High-Fidelity Polymerase	0.5	
dH_2O	27	

1 – Up to 10ng of template plasmid or DNA was added

Gels were formed from 50mL 1% (w/v) agarose in TBE buffer (90 mM Tris HCl, 90 mM Boric Acid, 2mM EDTA) and adding 2µg/ml ethidium bromide after the gel had cooled. DNA samples were mixed with 6x loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene-cyanol blue, 40% (w/v) sucrose in water) and run alongside either a 1kb+ or 1kb DNA ladder, also mixed with loading dye. as appropriate for the desired fragment of DNA. Electrophoresis was conducted at 100V for 45-60 minutes, depending on size and the resolution of band separation. The DNA was visualised using UV-light using a Molecular Imager Gel Doc Systems (Bio-Rad, US). Where DNA bands of interest were identified in agarose gels, these were removed using a scalpel and extracted using a QiaQuick Gel Extraction Kit (QIAGEN, Germany), as per the manufacturer's instructions. Isolated DNA was analysed using the Nanodrop 2000 UV-Vis Spectrophotometer.

2.14 DNA Sequencing

DNA was sequenced by Sanger sequencing using the Mix2Seq service provided by Eurofins (France). Test primers were added to sterile dH_2O, and DMSO was added to a final concentration of 5%, per the provider's instructions.

2.15 Constructing CRISPR/cas9 Genome Edited Strains

As described previously, the pCRISPomyces-2 system was utilised to perform genome edits (Cobb, Wang and Zhao, 2015). Protospacers for use in the single guide RNA (sgRNA) were designed to be approximately 20bp in length so that the last 15 nucleotides, including the NGG sequence, were unique to the targeted region to minimise off-target effects. These were ordered from IDT (US) as oligos before being annealed by heating to 95°C for 5 minutes, followed by ramping to 4°C at 0.1°C/second. Annealed protospacers were assembled into the BbsI cut site in the pCRISPomyces-2 vector using golden gate assembly, as described in Section2.8, and the assembled vector was digested using XbaI, as per Section 2.8. A 2kb homology repair template, formed from 1kb on either side of the target region, was then assembled into the vector using Gibson assembly, described in Section 2.8. The final vector was cloned in *E. coli* and, after being isolated, confirmed by PCR, detailed in Section 2.12, and sequenced, detailed in Section 2.14. Once confirmed, the vector was transformed into the desired actinomycete strain by conjugation from *E. coli* ET12567/pUZ8002, a non-methylating strain, as per Section 2.11. After deletion, mutants were plated on SFM lacking antibiotic selective pressure at 37°C for multiple generations to enable the loss of the pCRISPomyces-2 plasmid.

To confirm the deletion, two PCR products were generated using primer pairs 1F/1R and 2F/R2 from genomic DNA, extracted as per Section 2.8, or lysed mutant colonies, as described in Section 2.12, visualised in Figure 8. This allows PCR confirmation of mutants that still contain the pCRISPomyces-2 plasmid as the primers bind outside of the repair template, instead binding the genomic DNA on either side of the targeted gene. When these products are run down a gel, as per Section **Error! Reference source not found.** and visualised in Figure 9, this results in a wild-type

band and a band higher up the gel corresponding to the wild-type lacking the deleted region.

Figure 8 - Positioning of the 1F, 1R, 2F, and 2R Primers used to Confirm Deletion by CRISPR/cas9

Figure 9 - Example Gel Comparing Ex-Conjugants to Empty Controls for a Deletion of Approximately 1.5kb in *Streptomyces* FG4. The four leftmost columns contain DNA fragments approximate 2000bp longer than the middle four empty vector fragment, the same size as the DNA fragment inserted – suggesting the intended insertion has occurred.

2.16 Complementation of Deleted Genes

The deleted region, with a 500bp extension on either side, was amplified using PCR, as described in Section 2.13, before being assembled into pSS170 using Gibson assembly and then amplified by transforming into *E. coli* dH5α Section as described in Section 2.9. A colony PCR was performed to confirm the assembled plasmid, as per Section 2.10. Successfully confirmed plasmids were transformed into *E. coli* ETpUZ, as per Section 2.9, and cultivated on DNA agar supplemented with chloramphenicol,

kanamycin and hygromycin for one or two days, as required, at 37°C. The resulting cells were then conjugated into the actinomycete, as described in Section 2.11.

2.17 Chemical Extractions from Agar Plates using Solvents

Actinomycetes were cultivated on agar plates for the time specified in Section 3.1 at 30°C. For initial extractions, a single plate was used whilst for larger 1L of media was poured, and all resultant plates were used. The agar was diced into 1cm chunks using a razor blade before being placed into a 1L Duran, which was then filled with the solvent used. These were wrapped in three layers of blue roll to protect from UV light and then placed on a reciprocating shaker at 120rpm for two hours. This product was then poured into centrifuge tubes and centrifuged at 4000rpm for 10 minutes to remove the bulk waste, and the supernatant was poured off. The solvent was then evaporated using a Rotovap until the total volume could fit inside an EZ-2 Elite 3.0 Personal Evaporator (Genevac, UK) on the HPLC-Lyo setting. For water extractions, the Rotovap step was skipped, and instead, the sample was split into multiple Genevac batches, which were then recombined. The samples were then resuspended in the minimal amount of acetonitrile for further analysis. Sample weights, and thus, concentrations were not standardised between strains. This limits the ability for extracts from different samples generated through this method to be compared. Within a strain, concentration was maintained between samples.

2.18 Freeze/Thaw Chemical Extractions from Agar Plates

Agar plates were inoculated with the selected actinomycete for the time specified in Section 3.1 at 30°C. The resulting plates were diced into 1cm chunks using a razor blade and placed into a Duran, which was placed at -20°C overnight. These were then removed from the freezer and allowed to defrost, transferring the product into centrifuge tubes and centrifuged at 4000rpm for 10 minutes to remove the bulk waste, and the supernatant poured off. The water was then evaporated using an EZ-2 Elite 3.0 Personal Evaporator (Genevac, UK) on the HPLC-Lyo setting and the product was resuspended in a minimal amount of acetonitrile for further analysis.

2.19 Fractionation of Extracts

To perform fractionation, 750µL of extract – the maximum allowed sample volume - was injected into an Agilent 1260 Infinity II (Agilent, USA) whilst using the manufacturer's recommended settings for preparatory HPLC. A C-18 reverse-phase HPLC column was used with a gradient from 10-90% methanol supplemented with 1% V/V formic acid and acetonitrile supplemented with 1% V/V formic acid. The output from the column was collected in a vial, and the vial changed every 30 seconds. The collected output in these vials was then dried using an EZ-2 Elite 3.0 Personal Evaporator (Genevac, UK) on the low BP setting and then resuspended in 750µL of acetonitrile for further analysis.

2.20 LC/MS Analysis

Analytical LC/MS was performed using an Agilent 1260 Infinity II (Agilent, USA), using the setting recommended by the manufacturer. For preparatory HPLC, a Kinetex 5µm 250x21.2mm C-18 column was used with a gradient from 10-90% methanol supplemented with 1% V/V formic acid and acetonitrile supplemented with 1% V/V formic acid over a 15-minute run. For analytical HPLC, the run length was reduced to 10 minutes and the column replaced with a Kinetex 5µm 100x4.6mm C18 column. Both positive and negative ionisation modes were used to collect mass spectrometry data in preparatory and analytical HPLC, with an m/z range of 200-2000 and maximum ion injection time of 20ms.

2.21 LC/MS/MS Analysis

A Q Exactive Hybrid Quadrupole-Orbitrap was used with the settings recommended by the manufacturer. A Kinetex 1.7µm 50x2.1mm C18 column (Phenomenex, Denmark) was used, preceded by a SecurityGuard ULTRA UHPLC 2.1mm cartridge to protect the column from debris. A gradient from 10-90% methanol supplemented with 1% V/V formic acid and acetonitrile supplemented with 1% V/V formic acid over a 15-minute run. Both positive and negative ionisation modes were used to collect mass spectrometry data with an m/z range of 200-2000 and a maximum ion injection time of 35ms.

2.22 Biological Assays of Extracts by Liquid Dropping

Indicator strains of *B. subtilis, E. coli* or *C. albicans* were incubated overnight in 10mL LB broth at 37°C and 220rpm. These were then used to inoculate 100mL of soft-LB medium. 20mL of inoculated media was added to a 70cm^2 petri dish and allowed to set. Once set, 10µL of up to 7 different samples, extracts or fractions were carefully placed onto the top of the media and allowed to dry. 2µL of the appropriate antibiotic was used as a positive control and 10µL of the solvent the samples were suspended in as a negative. The concentrations of these samples were not standardised across conditions. An additional negative control of uninoculated media, extracted or fractionated in the same manner as the samples, was also tested. These were then placed into an incubator for 30°C overnight and inspected for signs of inhibition caused by the samples.

2.23 Biological Assays of Extracts by Disk Diffusion

Square plates of inoculated media were prepared in the same manner described in Section 2.22. 6mm filter paper disks were produced using a standard office hole punch and sterilised by autoclaving. These disks were soaked in 10µL of the sample, or fresh solvent for negative controls, before being placed onto the plate using sterile tweezers. Additionally, negative controls of uninoculated media, extracted or fractionated in the same manner as the samples, were tested. These were then placed into an incubator for 30°C overnight and inspected for signs of inhibition caused by the samples.

2.24 Analysis of the Effects of Formic Acid on Extracts

Formic acid was added to a vial containing 50µL of extract to a final concentration of 0.1% V:V. The sample was left at room temperature, with the vial lid removed, for 15 minutes, next to a vial containing the unmodified extract. The contents of both vials were then assessed for their biological activity, as described in Section 2.22.

2.25 Analysis of Effects of C-18 Column Material on Extracts

25mL of acetonitrile was passed through a Sep-Pak C-18 Cartridge (Waters, US). 50µL of the extract was added to the top of the Sep-Pak, and 10mL of acetonitrile passed

through the Sep-Pak, with the flow-through collected. Then, 10mL of ethyl acetate was passed through the Sep-Pak, and the effluent was collected. The two fractions were then dried using an EZ-2 Elite 3.0 Personal Evaporator (Genevac, UK) on its HPLC-lyo setting until the resulting powder was dry. The powder was then resuspended in 50µL of the same solvent that the extract was initially made in, and the biological activity was tested as per Section 2.22.

3 Genome Mining 18 Tropical Ant-Associated Actinomycete Strains

The discovery of natural products from actinomycetes has historically been relatively easy under laboratory conditions, leading to many clinically-relevant compounds. However, these comparatively easy-to-find antimicrobials produced by actinomycetes have now mostly been exhausted, and we face problems with rediscovery. This can be overcome by identifying actinomycetes from underexplored environments and using pleiotropic methods to activate smBGCs that are cryptic under normal laboratory conditions. Ocean sponges and desert environments have been explored as sources of novel actinomycete strains, as have insect symbionts—*Amycolatopsis sp.* M39 was isolated from a colony of the fungal farming termite *Macrotermes natalensis,* that was found to produce the antimicrobial macrotermycin (Beemelmanns *et al.*, 2017). Additionally, the antibiotic formicamycin was found to be produced by *S. formicae* isolated from a colony of the fungus-growing ant *Tetraponera penzigi* colony, demonstrating the ability for insect symbionts to both be novel organisms and for these organisms to encode novel antimicrobials (Qin *et al.*, 2017b). Once isolated, pleiotropic techniques can be utilised to unlock cryptic BGCs in these novel strains. Many BGCs are inactive under laboratory conditions as most specialised actinomycete media are designed to optimise the growth of the bacterium*,* not replicate the environmental conditions that stimulate the production of many of these costly secondary metabolites. By cultivating actinomycete strains on various media, some of which provoke stress responses in the bacteria, you improve the probability that the smBGC is activated on at least one of them. Ingredients in the medium can be used to effect global regulation, for example, by changing the carbon source between relatively easy to metabolise sugars, such as glucose, to more complex carbon sources, such as maltodextrin or porridge oats. One could also alter the nitrogen source, for example, between corn steep liquor and peptone. Other alternatives include altering metal ion concentrations, osmotic pressure or supplementing the medium with signalling molecules, such as N-acetyl-glucosamine.

In this chapter, the high-quality genome sequences of the 18 cultivatable actinomycete strains were analysed using antiSMASH to predict the BGCs they encode, with the expectation of some strains being predicted to encode cryptic antimicrobial-encoding smBGCs. For the rare actinomycetes, this also provided an opportunity to explore how biosynthetically rich

these strains may prove to be. The biological activities of these strains were assessed by challenging them with human pathogens upon a variety of media to unlock cryptic smBGCs, as it was predicted that some strains may only express antimicrobial activity when cultivated under specific, but unknown, conditions. This activity was compared to the predicted BGCs to identify potentially novel antimicrobials. Finally, an analysis of the genealogy was performed to determine how related these strains were to each other and to the model organism *S. coelicolor* A(3)2 to determine how they compare to previously discovered organisms. These 18 strains represent the entire collection of ant-associated actinomycetes available to the author at the commencement of the project, where high-quality whole genome sequences had been obtained.

3.1 Sporulation of Isolated Strains

Most secondary metabolites produced by actinomycetes are produced alongside the erection of aerial hyphae, which occurs just before sporulation (Seipke, Kaltenpoth and Hutchings, 2012). The time to sporulation, and thus areal hyphae erection, can vary between strains – particularly with rare actinomycetes for which growth media has not been optimised. To determine the duration of cultivation required for the strains to sporulate, each strain was plated on Soya Flour Medium (SFM), a solid agar medium that has been optimised for the growth of *Streptomyces*. As presented in Table 7, most strains took 8-10 days to grow and sporulate, but five strains – all rare actinomycetes - took 18-20 days on SFM, whilst two could not be revived from storage. The strains were then grouped into either a 10-day or 20-day growth period, depending on which more closely reflected their time to sporulation. This time was allowed to pass between inoculation and bioassays being performed regardless of the colony's sporulation state at the end of the growth period. Not all strains sporulated on all media by the end of the growth period. This indicates that it was likely that different strains were at different lifecycle stages when tested upon the same media, limiting the ability to compare between strains. Although only optimising growth period on SFM may have meant sub-optimal growth conditions were utilised for other media types, there was insufficient time for each actinomycete strain to be optimised for each media. Less media would have been able to be tested if optimisation on each media had occurred. This may have limited the chemical diversity identified on each medium, but the additional number of media would have increased chemical diversity, potentially balancing this effect. Additionally, by using

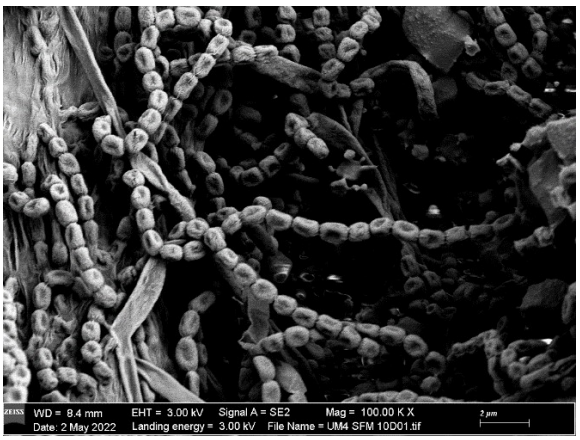
arbitrary growth periods, it allowed for streamlined sample processing as all strains could be tested on the same day – which had the additional benefit of ensuring all samples were tested on the same day and under the same environmental conditions.

SEM images were produced to confirm that sporulation had occurred on SFM, as described in Section 2.5. In order to maintain spore structure for imaging, the sublimation step had to be optimized as the sublimation method recommended by the manufacturer (-90°C for 4 minutes) resulted in spores collapsing due to desiccation. Conversely, when sublimation methods used a lower temperature for a similar duration (-100°C for 4 minutes), traces of ice remained on the sample, polluting the image. Given the time involved in preparing samples for the SEM, it was determined that performing the sublimation at -90°C for 2 minutes could balance the removal of ice particles with maintaining spore structure. For some samples, some ice particles remained, but these were few enough to be able to take adequate images. A selection of these SEM images, containing one from each strain, is shown in Figure 10, with further images in Appendix 7.1. These images were taken after cultivating the relevant actinomycete on SFM for 10 or 20 days, as described in Table 7. Individual spores can be seen in these images, and many connective proteins between spores are also visible. In some images, it is possible to identify both sporulated and vegetative cells, possibly due to the division of labour within a *Streptomyces* colony where the vegetative cells are responsible for antimicrobial production whilst others act as spore formers (Z. Zhang *et al.*, 2020). It is believed that the SEM images of *Tsukamurella, Jiangella* and *Agrococcus* reported here are the first SEM images of spores produced by these strains. Most rare actinomycetes studied here did not have significant morphological differences from *Streptomyces* when observed under SEM. Both *Amycolatopsis* UM15 and FG22 strains produced spores less distinct than is observed in *Streptomyces* colonies. This is seen ubiquitously across the sample, as seen in Figure 11, although the relative amount of fully differentiated spores varies in different regions of the sample. Areas with less distinct spores also showed a more significant amount of desiccation. This could be because these spores are immature and are so more susceptible to desiccation, or it could be that desiccation is responsible for the spores becoming less distinct from each other. Further study would be needed to determine which attribute, if any, is causal. Of additional interest is the unusual spiral structures formed by the spore chains of *Pseudonocardia* P1 that were seen in multiple places around the sample. This was the only

strain to produce spore chains in spiral structures in significant quantities, although *Streptomyces* KY2 also had a small number.

Table 7 - Time Taken for Strains to Grow to Sporulation on SFM

Strain Designation	Time to Sporulate on SFM (Days)	Time allowed in bioassays (Days)
Pseudonocardia UM4	10	10
Pseudonocardia UM14	8	
Pseudonocardia UM9	9	
Pseudonocardia P1	10	
Pseudonocardia PS2	Failed to Grow	N/A
Streptomyces KY2	7	10
Streptomyces B2	8	
Streptomyces FG4	7	
Streptomyces FG7	8	
Streptomyces A7	10	
Streptomyces KY4	9	
Streptomyces FG1	8	
Streptomyces KY1	10	
Amycolatopsis UM15	17	20
Amycolatopsis FG22	20	
Agrococcus A6	20	
Tsukamurella FG11	19	
Nocardiopsis E5	Failed to Grow	N/A
Tsukamurella E7	19	20
Jiangella S1	20	



A

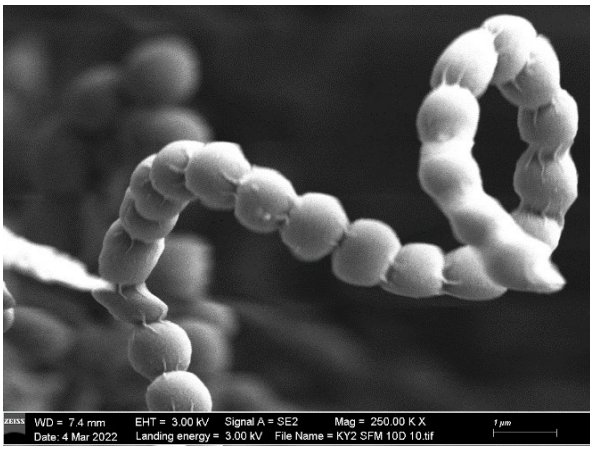
B


WD = 9.4 mm EHT = 3.00 kV Signal A = SE2 Mag = 150.00 K X
Date: 28 Mar 2022 Landing energy = 3.00 kV File Name = UM9 SFM 10D10.tif

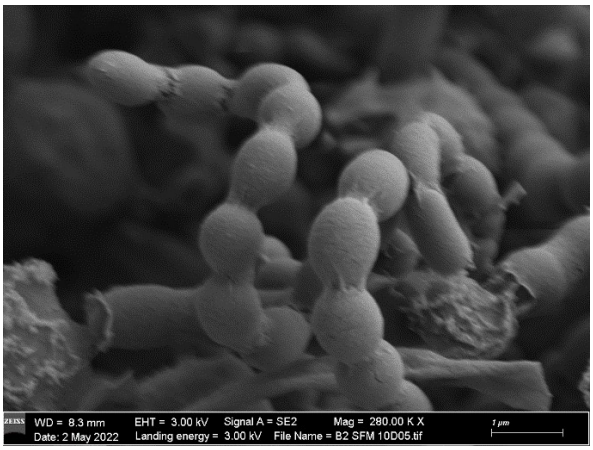
C


WD = 8.5 mm EHT = 3.00 kV Signal A = SE2 Mag = 80.00 K X
Date: 2 May 2022 Landing energy = 3.00 kV File Name = P1 SFM 10D01.tif

D


WD = 7.4 mm EHT = 3.00 kV Signal A = SE2 Mag = 250.00 K X
Date: 4 Mar 2022 Landing energy = 3.00 kV File Name = KY2 SFM 10D 10.tif

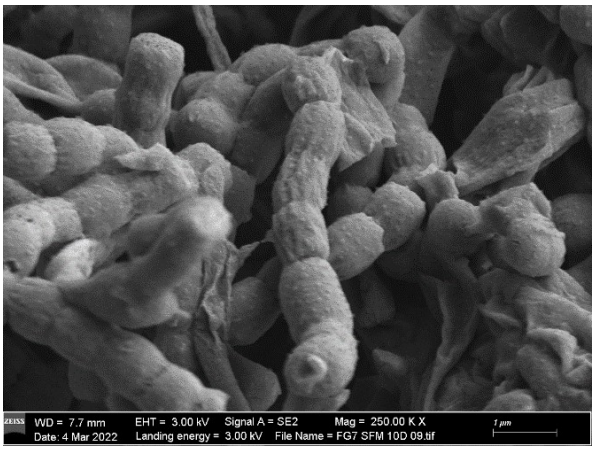
E


WD = 8.3 mm EHT = 3.00 kV Signal A = SE2 Mag = 280.00 K X
Date: 2 May 2022 Landing energy = 3.00 kV File Name = B2 SFM 10D05.tif

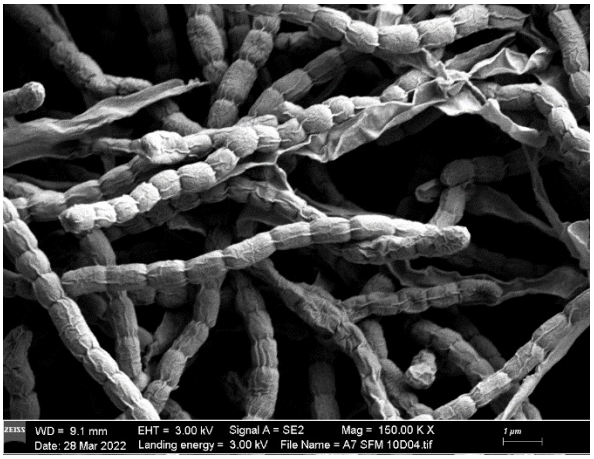
F


WD = 8.1 mm EHT = 3.00 kV Signal A = SE2 Mag = 250.00 K X
Date: 4 Mar 2022 Landing energy = 3.00 kV File Name = FG4 SFM 10D 11.tif

G


WD = 7.7 mm EHT = 3.00 kV Signal A = SE2 Mag = 250.00 K X
Date: 4 Mar 2022 Landing energy = 3.00 kV File Name = FG7 SFM 10D 09.tif

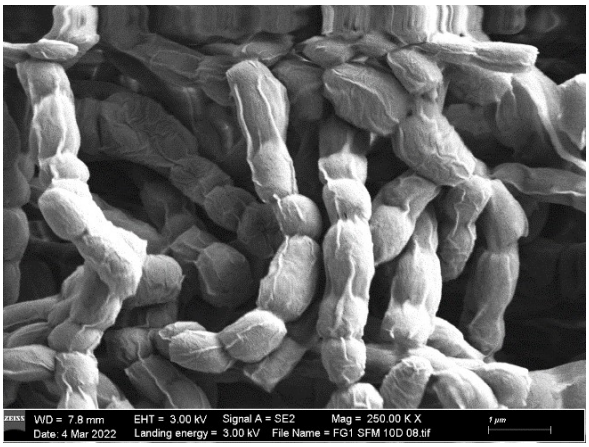
H


WD = 9.1 mm EHT = 3.00 kV Signal A = SE2 Mag = 150.00 K X
Date: 28 Mar 2022 Landing energy = 3.00 kV File Name = A7 SFM 10D04.tif

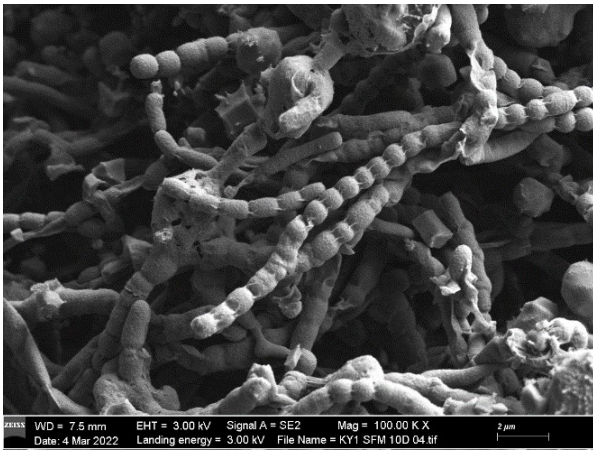
I


WD = 7.7 mm EHT = 3.00 kV Signal A = SE2 Mag = 150.00 K X
Date: 4 Mar 2022 Landing energy = 3.00 kV File Name = KY2 SFM 10D 05.tif

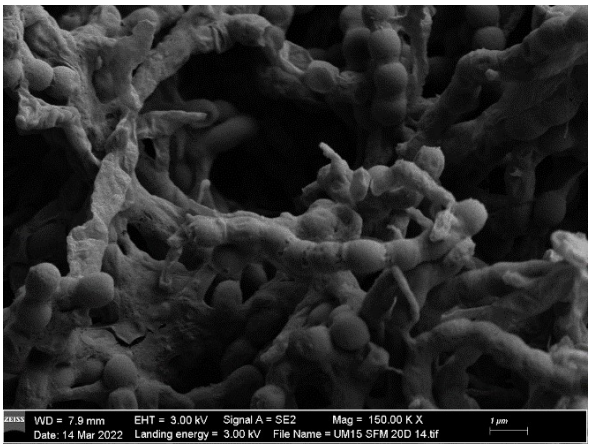
J


WD = 7.8 mm EHT = 3.00 kV Signal A = SE2 Mag = 250.00 K X
Date: 4 Mar 2022 Landing energy = 3.00 kV File Name = FG1 SFM 10D 08.tif

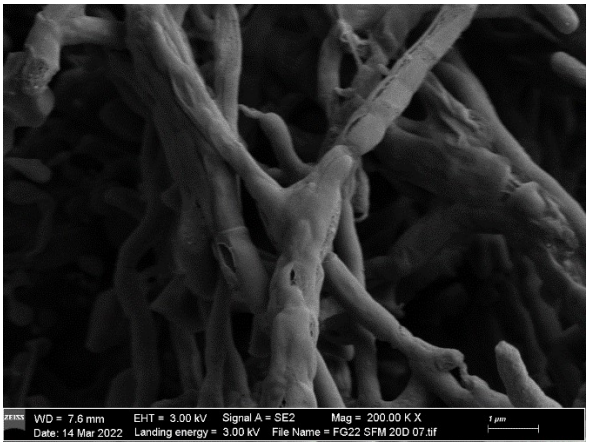
K


WD = 7.5 mm EHT = 3.00 kV Signal A = SE2 Mag = 100.00 K X
Date: 4 Mar 2022 Landing energy = 3.00 kV File Name = KY1 SFM 10D 04.tif

L


WD = 7.9 mm EHT = 3.00 kV Signal A = SE2 Mag = 150.00 K X
Date: 14 Mar 2022 Landing energy = 3.00 kV File Name = UM15 SFM 20D 14.tif

M


WD = 7.6 mm EHT = 3.00 kV Signal A = SE2 Mag = 200.00 K X
Date: 14 Mar 2022 Landing energy = 3.00 kV File Name = FG22 SFM 20D 07.tif

N

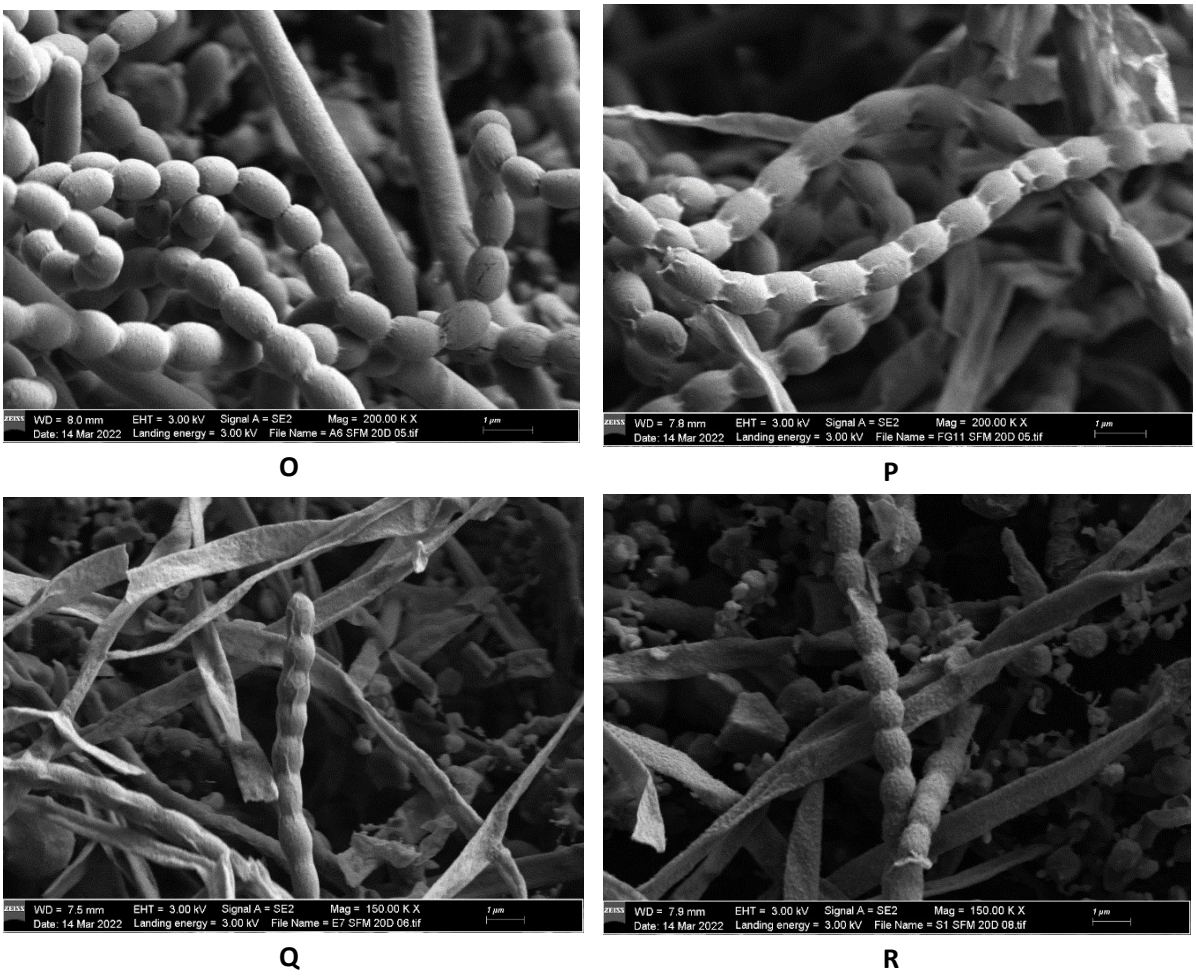


Figure 10 - SEM images of the 18 cultivatable strains after having been grown on SFM for the period described in Table 7. A – *Pseudonocardia* UM4, B - *Pseudonocardia* UM14, C – *Pseudonocardia* UM9, D – *Pseudonocardia* P1, E - *Streptomyces* KY2, F – *Streptomyces* B2, G – *Streptomyces* FG4, H – *Streptomyces* FG7, I – *Streptomyces* A7, J – *Streptomyces* KY4, K – *Streptomyces* FG1, L – *Streptomyces* KY1, M – *Amycolatopsis* UM15, N – *Amycolatopsis* FG22, O – *Agrococcus* A6, P – *Tsukamurella* FG11, Q – *Tsukamurella* E7, R – *Jiangella* S1. These images show that spore formation has occurred at the tested timepoint, although in some cases some hyphae have not completed segregation – images D, O and Q.

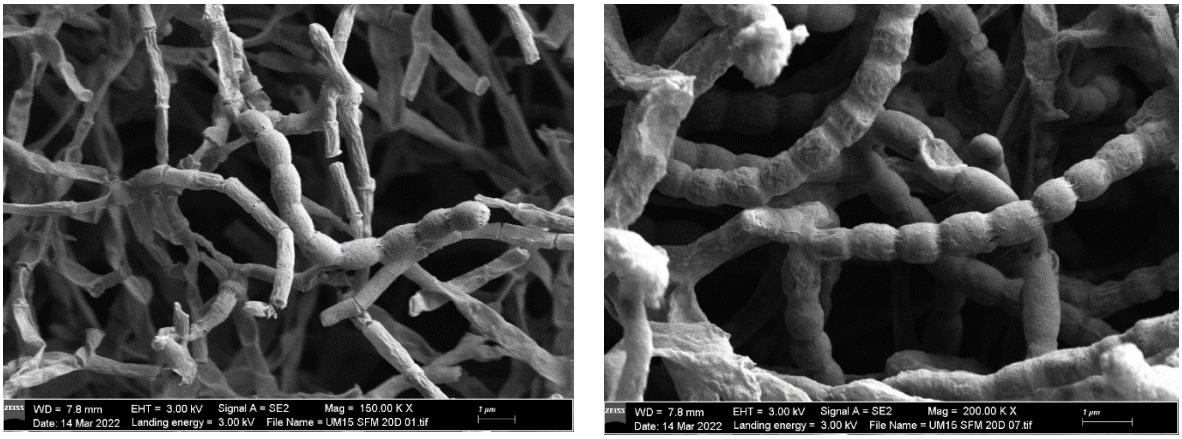


Figure 11 - SEM Images of Two Distinct Regions of *Amycolatopsis* UM15. In the left image, spores are less distinct from each other than in the right image. There is also increased evidence of desiccation, which may partially explain this observation or may be a result of immature spores being more susceptible to desiccation.

3.2 Determining the biological activity of isolated strains

To determine the bioactivity of the strains isolated from fungus-growing ant colonies, a series of bioassays across 31 media conditions were conducted in the manner described in Section 2.6. Due to the limited variety of actinomycete strains available, it was decided to use such a broad range of media to maximise the potential for chemical diversity and whilst balancing the workload. As part of reducing the workload required to complete the experiments, these were performed in duplicate rather than triplicate to allow for a wider variety of media to be tested. If one duplicate demonstrated activity and the other did not, it was assumed to be inactive to reduce workload and allow a focus on strains that were consistently demonstrating bioactivity. Increasing the variety of media used would have been appropriate if a sub-set of the strains had been selected for investigation, however this would mean that other strains would have been left unstudied. Given that it is less likely for a single strain to encode a larger number of different antimicrobials than 18 strains, studying all 18 was deemed more likely to elicit interesting chemistry. Initially, media that induces sporulation in actinomycetes, such as SFM, was used to determine bioactivity and that sporulation occurred under laboratory conditions. Media other than SFM was used to expose the actinomycete strains to various conditions, including; alternate carbon or nitrogen sources, osmotic stress and metal ion concentrations, with the intent of inducing global regulators in actinomycetes to affect secondary metabolite production. In some cases, one medium was designed to induce multiple stress responses simultaneously, for example, LB – a widely used growth medium, which uses yeast extract as a carbon and nitrogen source or SM12, which uses high

concentrations of glucose to induce osmotic stress and uses a mix of peptone, beef extract powder and yeast extract as nitrogen sources. Some media varieties were supplemented with compounds, such as GluNAc, which mimic signalling molecules found in the environment or with sub-inhibitory concentrations of streptomycin. GluNAc supplementation was performed in SFM, which provides relatively high levels of nutrients to the actinomycete and in minimal media, which provides only a very basic amount to allow the actinomycete to survive, as GluNAc has been demonstrated to have different effects on the phenotype of *Streptomyces* depending upon nutrient availability. GluNAc is produced by the autolytic degradation of the mycelium and high concentrations as a checkpoint for antibiotic production by interacting with the repressor DasR and preventing it from binding to DNA (Rigali *et al.*, 2008). Streptomycin supplementation was performed at 2µg/mL and 10µg/mL concentration, compared to 50µg/mL usually used to inhibit microbial growth. These sub-inhibitory concentrations were selected to encourage antibiotic-induced genetic mutations, which may regulate gene expression by modulating rRNA and RNA polymerase. Mutations resulting in streptomycin resistance are most commonly associated with the *rpsL* gene, encoding the 30S ribosomal protein S12, which is involved in tRNA selection at the A site, and with S-Adenosyl methionine-dependent 16S rRNA methyltransferase, encoded by *rsmG*. Some of the spontaneous mutations in *rpsL* and *rsmG,* caused by exposure to low concentrations of streptomycin, result in enhanced streptomycin resistance, hyper-accurate translation and antibiotic overproduction (Valle *et al.*, 2002; Ochi, 2016). Other media, such as YEME (Blodgett *et al.*, 2010) and SPY (Imai *et al.*, 2015) were selected due to their use in previous studies where they successfully elicited antimicrobial activity. Further, two examples of media from the International *Streptomyces* Project – ISP2 and 4 – due to their historical application in similar studies. ISP 3 was not used as it has a similar ingredient mix as IMA, which is more commonly used in the author's laboratory and ISP 5 to minimal. In addition to containing only complex carbon and nitrogen sources, YP and YPD were included due to their recent use in studies looking at *Streptomyces* exploration, both to identify if any of the strains studied here could undergo exploration and for the potentially different secondary metabolite expression during the exploration growth phase (Jones *et al.*, 2019). All of the media prefixed with SM were media historically used by the author's laboratory. They range in carbon sources, pH and other stress factors, such as SM19 and 30 which contain the same base ingredients – tomato paste and oat flour – to provide complex carbon source but have significantly different pH

(pH7.0 vs pH4.5). Other SM media included SM12, which provides significant osmotic pressure but a readily available carbon source in the form of glucose. Section 2.2 contains the complete list of media used in attempts to elucidate bioactivity.

All strains were screened against *B. subtilis* 168, *E. coli* NCTC 12923 and *C. albicans* ATCC MYA 2876 as examples of Gram-positive, Gram-negative and fungal human pathogens. The zone of inhibition around the actinomycete colony was measured before the strain was assigned the category 'P' (potent) if the zone of inhibition was over 10mm, 'S' (some) if a zone of inhibition was present but smaller than 10mm or 'N' (none) if no zone of inhibition was observed. If the actinomycete did not grow or did not sporulate on the growth medium, it was assigned an 'F' (failed). Examples of how these assignments were made can be seen in Figure 12.

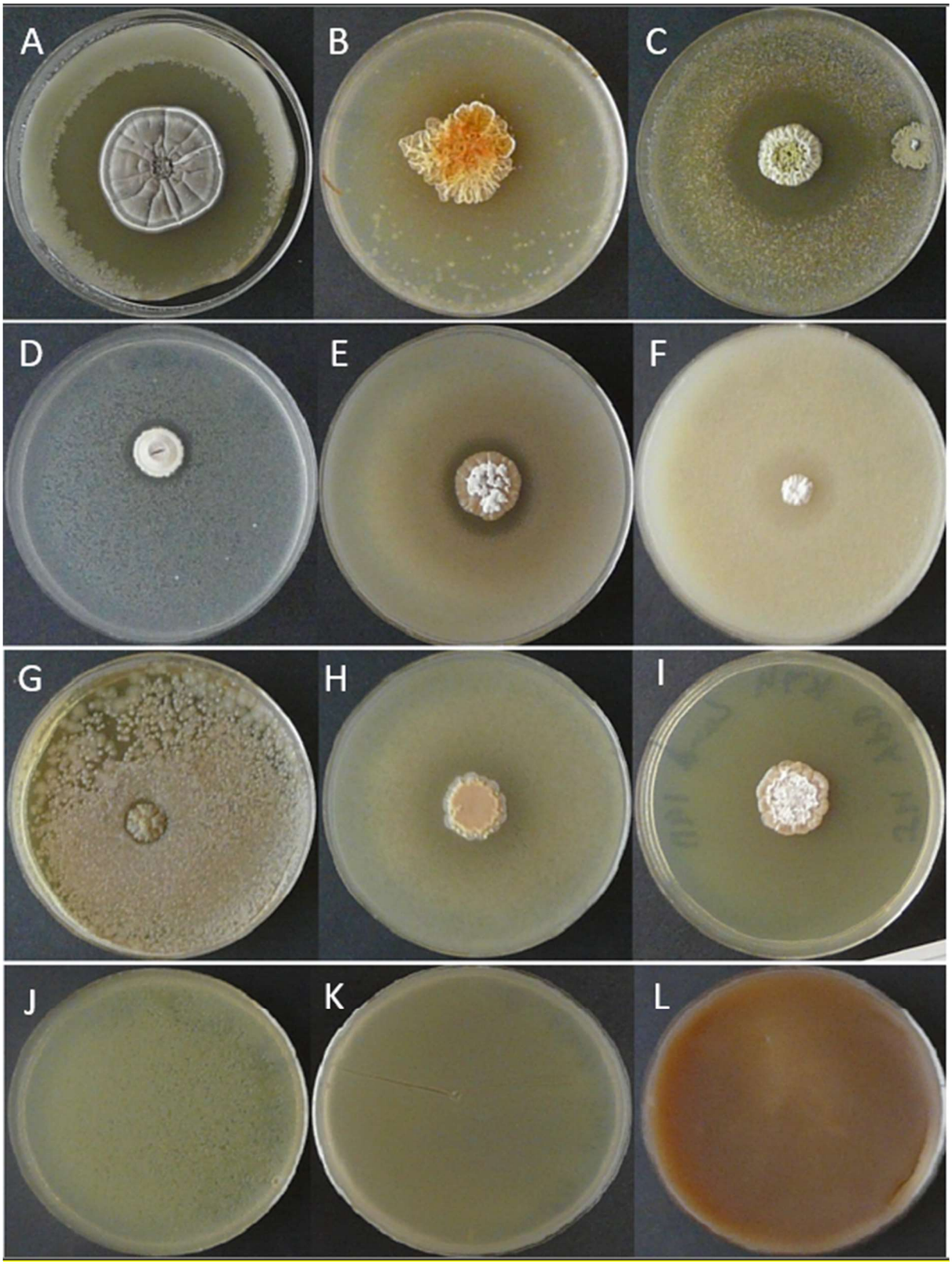


Figure 12 - Examples of Bioassay Results Categorisation. Letters A-C represent tests given the designation 'P' as a zone of inhibition of 10mm or larger was observed, D-F 'S' as a zone of inhibition below 10mm was observed, G-I 'N' as no zone of inhibition was observed and J-L 'F' if the actinomycete failed to grow on the medium. A; *Streptomyces* FG4 on MYM vs *B subtilis,* B; *Amycolatopsis* FG22 on SM7 vs *E. coli*, C; *Streptomyces* KY2 on FML vs *B. subtilis*, D; *Streptomyces* A7 on MinNAG vs *C. Albicans*, E; *Streptomyces* A7 on YPD vs *B. subtilis,* F; *Pseudonocardia* P1 on SFMNAG vs *C. albicans*, G; *Streptomyces* B2 on SPY vs *B. subtilis*, H;

Streptomyces FG4 on YP vs *B. subtilis*, I; *Streptomyces* KY4 on YPD vs *C. albicans*, J; *Agrococcus* A6 on YPD vs *C. albicans*, K; *Pseudonocardia* UM14 on SM15 vs *B. subtilis*, L; *Amycolatopsis* UM15 on SM18 vs *C. albicans*.

Table 8 summarises the bioactivity observed, broken down by actinomycete strain, across the 31 media the strains were grown on. All 18 strains subjected to pleiotropic attempts to elicit bioactivity demonstrated activity against *B. subtilis* on at least one growth medium. The two *Amycolatopsis* strains – UM15 and FG22 – demonstrated activity on the broadest range of media across all pathogens (UM15 vs; *B. subtilis* 18*, E. coli* 9*, C. albicans* 10. FG22 vs; *B. subtilis* 16*, E. coli* 9*, C. albicans* 11), although *Streptomyces* A7 also inhibited *B. subtilis* across a similarly wide number of media (16), it only inhibited *E. coli* on one medium and *C. albicans* on three. The *Streptomyces* strains can be broadly fit into two camps – those that inhibited *B. subtilis* across a wide variety of media (KY2, 12; FG4, 12; A7, 16; FG1, 13) and those that only expressed *B. subtilis* inhibition on a narrow range (B2, 4; FG7, 3; KY4, 1; KY1, 5). Interestingly, this second group of *Streptomyces* showed bioactivity against *B. subtilis* on a similar range of media as the rare actinomycetes (except *Amycolatopsis* UM15 and FG22), with none showing activity on more than five different media.

E. coli inhibition was more challenging to elicit than *B. subtilis*¸ with two strains (*Amycolatopsis* UM15 and FG22) demonstrating activity on nine media, but no other strains demonstrating *E. coli* inhibition on more than five media (*Streptomyces* FG4). Additionally, eight strains (*Pseudonocardia* UM14, UM9; *Streptomyces* B2, KY4, KY1; *Tsukamurella* FG11, E7) showed no ability to inhibit *E. coli* on any medium. All strains that inhibited *E. coli* did so on a narrower range of media than *B. subtilis* inhibition was observed. There may be multiple antimicrobials encoded in the genome of the strain, but only a subset can inhibit *E. coli,* and the different growth conditions induce the production of the different encoded antimicrobials. It is also possible that only one antimicrobial is encoded in the genome, but it is only produced in concentrations high enough to inhibit *E. coli* under certain conditions.

Although bioactivity towards *C. albicans* was observed in every strain tested, activity was harder to elicit than activity against *B. subtilis*, with several strains (*Pseudonocardia* UM14, *Streptomyces* FG7 and *Agrococcus* A6) only exhibiting the ability to inhibit *C. albicans* growth on a single medium. Given the association with fungus-growing ants, and the need to defend against *Escovopsis* invasion, it is perhaps unsurprising that antifungal activity would be observed in all strains. This may suggest a tendency for the ant colony to select for

actinomycete strains able to inhibit fungal growth, although further study would need to be conducted to confirm this. The *Amycolatopsis* strains UM15 and FG22 demonstrated bioactivity against *C. albicans* on the widest variety of growth media, inhibiting *C. albicans* growth on 10 and 11 different media, respectively. All strains showed activity against *C. albicans* independently from bacterial inhibition – activity was not always observed on the same media against *C. albicans* and the bacterial pathogens, suggesting the compounds produced are unlikely to be broadly cytotoxic and that different compounds are responsible for bacterial and fungal inhibition.

Table 8 - **Summary of Bioactivity Observed by Strain.** The numbers reported here are for conditions resulting in an S or P designation. All actinomycete strains demonstrated the ability to inhibit growth of *B. subtilis* on at least one medium, and in all cases activity was observed on *B. subtilis* more than *E. coli*. This suggests that in many cases different compounds were responsible for the antimicrobial activity, or that concentrations high enough to inhibit *E. coli* growth were produced on a smaller variety of media.

	Number of Media a Zone of Inhibition was Observed when Challenged by		
Strain Designation	***B. subtilis***	***E. coli***	***C. albicans***
Pseudonocardia UM4	5	3	4
Pseudonocardia UM14	2	0	1
Pseudonocardia UM9	5	0	3
Pseudonocardia P1	2	1	5
Streptomyces KY2	12	4	5
Streptomyces B2	4	0	1
Streptomyces FG4	12	5	7
Streptomyces FG7	3	0	1
Streptomyces A7	16	1	3
Streptomyces KY4	1	0	2
Streptomyces FG1	13	3	6
Streptomyces KY1	5	0	5
Amycolatopsis UM15	18	9	10
Amycolatopsis FG22	16	9	11
Agrococcus A6	3	1	1
Tsukamurella FG11	4	0	3
Tsukamurella E7	3	0	2
Jiangella S1	3	1	2

Table 9 contains the bioactivity observed by the 18 strains, broken down by medium. FML and SM20 both elicited activity by nine actinomycetes against *B. subtilis,* the most of any medium. FML further went on the elicit four strains to produce activity against *E. coli*, second

only to GYM+10µg/mL streptomycin (5), and five to inhibit *C. albicans,* beaten by SFM, GYM and GYM+2µg/mL streptomycin each of which elicited six actinomycetes to inhibit the fungus. FML contains a high glucose concentration (40g/L), potentially inducing a high level of osmotic stress on the cultured actinomycete whilst also providing a readily available carbon source. This alone cannot explain the wide number of actinomycetes demonstrating bioactivity as SM12 contains a higher concentration of glucose (50g/L), but fewer actinomycetes were active against *B. subtilis* (5), *E. coli* (3) and *C. albicans* (2). A bioassay from both FML and SM12 is shown in Figure 13. It seems unlikely that this is due to SM12 being a more difficult medium to grow on, as it did not have any strains that failed to grow, whereas FML had two strains. Another possible explanation for FML's ability to elicit activity is the complex nitrogen source, peptone (10g/L), used in the medium. Increasing the nitrogen source's complexity may have put the actinomycete under increased resource pressure, stimulating the production of antimicrobials to reduce competition from other organisms that may be present in the strain's natural environment.

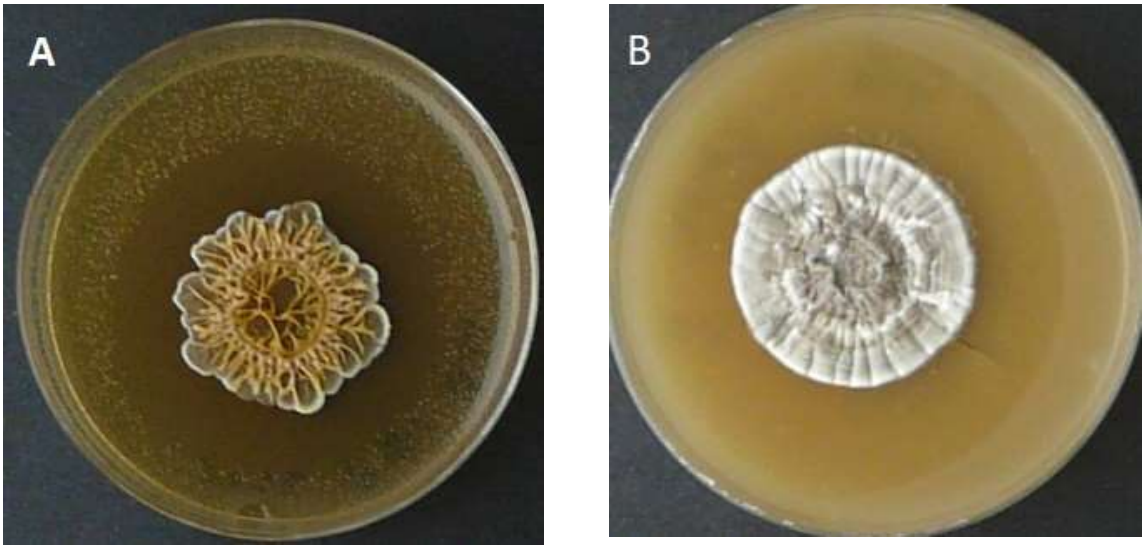


Figure 13 - Bioassays of A - *Streptomyces* FG1 on FML when Challenged by *B. subtilis* and B - *Streptomyces* FG4 on SM12 when Challenged by *E. coli*

SM20 contains a moderately high maltose concentration (20g/L), a disaccharide made from two glucose molecules connected by an α(1→4) bond. Assuming the actinomycete strain possesses a maltase enzyme, this can provide a readily available carbon source for metabolism, similar to the glucose in FML. SM12 also contains peptone (5g/L), as well as other complex nitrogen and carbon sources in the form of beef extract (5g/L) and yeast extract (3g/L). This combination of readily available carbon and complex nitrogen sources effectively

elicits antimicrobial production among the actinomycete strains tested in this work. This may be because this environment is a closer reflection of the ant carapace, from which these strains were isolated, given that some species of leafcutter ant are known to provide carbon in the form of glucose to the bacteria inhibiting their carapace (Currie *et al.*, 2006; Worsley *et al.*, 2021).

However, confounding this theory is the fact that SM12, which contains a high concentration of glucose (50g/L) as well as peptone (4g/L), beef extract (4g/L) and yeast extract (1g/L), proved less effective at eliciting antimicrobial production than SM20. SM12 and SM20 contain sodium chloride at similar concentrations (2.5g/L, 3.0g/L) and have a similar pH (7.6, 7.2), so it seems unlikely these factors can explain this difference in antimicrobial production. This is exacerbated by SM5, which elicited antimicrobial activity in relatively few actinomycete strains (*B. subtilis*; 4, *E. coli*; 0 and, *C. albicans*; 1) despite containing a readily available carbon source (glucose, 15g/L) and a complex carbon and nitrogen source (peptone, 20g/L; beef extract, 8g/L), as well as being a similar pH (7.2) to the highly eliciting SM20. Multiple characteristics of the medium are likely responsible for eliciting antimicrobial production in the actinomycete strains tested. This is consistent with previous understanding of *Streptomyces* secondary metabolism, whereby combinations of pleiotropic regulatory genes affect multiple BGCs (Ostash, 2021).

GYM+10µg/mL streptomycin proved the most effective medium at eliciting activity against *E. coli,* with five strains demonstrating the ability to inhibit it upon this medium. This is higher than the single strain shown to inhibit *E. coli* on GYM+2µg/mL and none on GYM without any supplementation. This suggests that the addition of sub-inhibitory concentrations of streptomycin can be effective at unlocking cryptic BGCs in actinomycetes, supporting previous findings. Interestingly, however, activity against *C. albicans* was elicited in more strains when cultivated upon GYM+2µg/mL (8) than GYM+10µg/mL (3) or GYM alone (6). This could be explained if the potential mutations to *rpsL* and *rsmG* reduced the ability of these strains to produce the antifungal when streptomycin concentrations were higher or if resources were diverted to streptomycin resistance mechanisms rather than antimicrobial production at the higher concentration. At lower concentrations, it may be that the streptomycin is acting more like a signalling molecule to elicit production rather than an antimicrobial agent.

Table 9 - Summary of Activity by Medium. The numbers reported here are for conditions resulting in an S or P designation, except the 'failed to grow' column, which returned an 'F' result. In the *B. subtilis, E. coli* and *C. albicans* columns, a green colour indicates a relatively high number of actinomycete strains inhibited that organism on that medium. Note that the colour scaling in each column is distinct from other columns. If one replicate for a given condition demonstrated activity whilst the other did not, it is included here as a inhibition being observed. FML and SM20 were able to induce the highest number of actinomycete strains to produce metabolites capable of inhibiting *B. subtilis*, with GYM+10µg/mL streptomycin and GYM+2µg/mL streptomycin inducing the most to exhibit *E. coli* and *C. albicans* activity respectively.

	Number of Strains a Zone of Inhibition was Observed when Challenged by			
	B. subtilis	***E. coli***	***C. albicans***	**Failed to Grow**
SFM	5	1	6	0
SFMNAG	5	2	4	0
SFMSB	4	1	3	0
YP	3	0	3	1
YPD	3	0	3	1
SPY	3	0	1	0
FML	9	4	5	2
MYM	4	1	4	0
YEME	5	1	3	0
IMA	2	1	1	0
GYM	6	0	6	0
Minimal	4	2	1	1
SM3	4	1	1	2
SM5	4	0	1	1
SM6	6	1	3	1
SM7	5	1	2	1
SM12	5	3	2	0
SM14	5	0	1	0
SM15	2	1	1	11
SM18	4	1	0	2
SM19	1	0	0	3
SM20	9	0	1	0
SM25	7	3	2	0
SM30	3	1	0	0
SM32	5	1	1	2
MinNAG	5	2	3	2
GYM+10µg/mL streptomycin	4	5	3	0

GYM+2µg/mL streptomycin	2	1	8	1
YP/C	2	1	0	0
ISP2	1	1	0	0
ISP4	1	0	0	0
SFM+CasAA	2	2	1	0

Eleven strains failed to grow on SM15 (*Pseudonocardia* UM4, UM14, UM9, P1; *Streptomyces* KY2, A7, KY4, FG1, KY1; *Jiangella* S1; *Amycolatopsis* UM15) more than any other growth medium tested in this work. SM15 contains 11.5g/L of casamino acid, initially the only medium here to contain casamino acids at any concentration. Casamino acids are a mixture of amino acids and the products from the acid hydrolysis of casein, providing a nitrogen source and a source of free amino acids for the bacteria being cultured. However, when casamino acids were added to SFM at the same concentration as in SM15, strains could grow effectively, suggesting that casamino acids alone were not the cause of these failures. Further study would be required to identify the cause of this apparent inhibition of actinomycete growth.

3.2.1 Biological Activity of Actinomycetes Co-Cultured with Pathogens in Liquid Medium

In addition to these solid media bioassays, liquid media bioassays where the actinomycete was co-cultured with a pathogenic organism were attempted. When bacteria are co-cultured together, they are allowed to interact chemically, whilst cells from each strain remain isolated from the other. This allows for signalling molecules to be shared between the two strains but ensure the two colonies remain axenic. Previous methodologies of performing similar experiments have required expensive, custom-blown glassware (Murray, Salih and Tucker, 2019). In an attempt to replicate this at a lower cost, a device was created with two 50mL centrifuge tubes placed neck-to-neck, separated by an 8kD dialysis membrane. The SFM broth (15ml) was added into each tube, one inoculated with the chosen pathogen and the other with the actinomycete. The topside of one of the lids of the tubes was removed, allowing a tube to be screwed onto the lid from each side. These were then secured using Parafilm to create a watertight seal. This is illustrated in Figure 14. The constructed device was then placed on a gel rocker at 10 rpm, with the device placed following the line of movement to ensure liquid moved from one half of the device to the other. The dialysis membrane prevents

either half of the device from being contaminated by cells originating from the opposite half but continues to allow small molecules to pass through. The actinomycete could potentially use these small molecules as signals to activate otherwise cryptic smBGCs encoding antimicrobials. Alternatively, by sharing resources, their depletion by the presence of the pathogenic organism may also elicit a response. This is all performed in liquid culture as it has previously been observed that some actinomycete strains activate different smBGCs when grown in liquid media rather than on solid, and that co-culture can elicit activity not observed when the actinomycete is grown in monoculture.

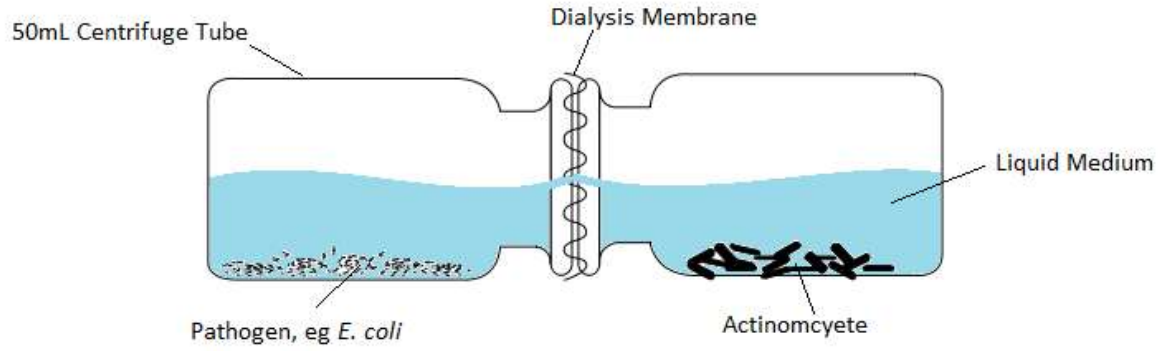


Figure 14 - Illustration of the liquid co-culture device created to allow liquid co-culturing of actinomycetes with human pathogens. Each vessel contains a single strain of bacteria, with the dialysis membrane allowing for transfer of molecules as the system is rocked but blocks cells from crossing.

Section 2.7 details the methodology used to determine if the liquid from this co-culture possessed biological activity for each of the actinomycete strains after cultivation for 14 days. Due to the complex nature of this method, it was only performed using liquid SFM, selected as SFM has been optimised for actinomycete growth and because this experiment was performed prior to the completion of the solid culture bioassays. Additionally, no replicates were performed in these initial trials. Table 10 summarises the results of the activity observed using this method. In general, strains were not as active when grown in this manner compared to the solid cultures, although the co-culture did elicit activity in several strains that did not demonstrate antimicrobial activity when grown in monoculture. Examples include *Pseudonocardia* P1 and *Streptomyces* KY4, from which co-culturing elicited activity against *C. albicans* and *B. subtilis,* respectively. No strains showed the ability to inhibit any pathogen when cultivated in liquid medium that they were not able to inhibit on solid SFM. As such, further work reported in this thesis was conducted using solid medium unless stated

otherwise. Further, it is worth noting that liquid culture and solid culture bioactivity is not comparable. Not only were cultivation times different between solid and liquid but the availability of nutrients, oxygen and pH are also different.

Table 10 - Results of Bioassays performed after growing actinomycetes in liquid culture, either as a mono-culture or when co-cultured with the target strain. A yellow 'S' designates a zone of inhibition below 10mm, a red 'N' no zone of inhibition, and orange 'F' if the actinomycete failed to grow on the medium. No zone of inhibition above 10mm was observed, so no 'P' designation was prescribed.

	Monoculture			Co-culture		
Strain	***B. subtilis***	***E. coli***	***C. albicans***	***B. subtilis***	***E. coli***	***C. albicans***
Pseudonocardia UM4	N	N	N	N	N	N
Pseudonocardia UM14	N	N	N	N	N	N
Pseudonocardia UM9	N	N	N	S	N	N
Pseudonocardia P1	N	N	N	N	N	S
Streptomyces KY2	S	N	N	S	N	N
Streptomyces FG4	S	N	N	S	N	N
Streptomyces B2	N	N	N	N	N	N
Streptomyces FG7	N	N	N	N	N	N
Streptomyces A7	S	N	N	S	N	N
Streptomyces KY4	N	N	N	S	N	N
Streptomyces FG1	N	N	S	N	N	S
Streptomyces KY1	N	N	N	N	N	N
Amycolatopsis UM15	S	N	S	S	N	S
Amycolatopsis FG22	S	N	S	S	N	S
Agrococcus A6	F	F	F	F	F	F
Tsukamurella FG11	N	N	N	N	N	N
Tsukamurella E7	N	N	N	N	N	N
Jiangella S1	N	N	N	N	N	N

3.3 Analysis of smBGCs Present in Strains

AntiSMASH 6.0.1 was used to identify smBGCs within strains that may encode for an antimicrobial by comparing the full genome sequences with previously-reported smBGCs known to encode for antimicrobials (Blin *et al.*, 2021). The antiSMASH output was manually reviewed to confirm the findings of the software, in particular where multiple smBGCs may have been classified as one smBGC. As presented in Table 11, the manually reviewed antiSMASH analysis identified 404 smBGCs across the 18 strains, with a total genome size of 129.98Mbp. All smBGCs detected had a high-level review to ensure antiSMASH had not combined multiple smBGCs into one entry. smBGCs were selected for a detailed manual

review if antiSMASH had determined a similarity to a known cluster of 80% or higher. If the compound encoded by the smBGC was predicted to be an antimicrobial, this was lowered to 70%. During the manual review, the discrepancies between the detected smBGCs and the previously-discovered smBGCs were analysed to determine if the similarity was in the core biosynthetic machinery of the smBGC, regulatory regions or elsewhere.

One example of a manually reviewed region was Region 1.8 of *Streptomyces* B2, which was predicted to have a 100% similarity to a polycyclic tetramate macrolactam (PTM) b/c/d smBGC in *S. griseus* – the product of which is discussed in Section 3.3.6 below. As shown in Figure 15, Region 1.8 was first compared to the PTM smBGC to ensure the structure of the cluster was similar, with genes with similar functions in the same relative positions. Each gene was then checked for its BlastP results within antiSMASH to ensure that the gene selected by antiSMASH had a high percentage identity and coverage, indicating that they likely produce similar proteins. Next, surrounding genes were inspected. In particular, regulatory genes and transport genes were looked for in the immediately adjacent genes, which suggested they were part of the Region's BGC, even if they were not included in the PTM smBGC as previously reported. In this example, *DKB33_19735*, *DKB33_19740* and *DKB33_19745*, the three genes immediately upstream of the predicted smBGC within Region 1.8, were predicted to encode a permease enzyme, involved in chemical efflux, a LysR family transcriptional regulator and IF-2 respectively. IF-2 is a nucleotide binding site that can bind the regulator guanosine 3',5'-(bis) diphosphate (ppGpp), typically found in higher concentrations when the bacterium is under stress, which may suggest that Region 1.8 is cryptic when grown in normal laboratory conditions (Milon *et al.*, 2006). This suggests that these three genes are related to the production of PTM in Region 1.8. Other genes, such as with *DKB33_19750* – predicted to encode a deacetylase enzyme, further upstream from *DKB33_19745* may have been part of the smBGC in Region 1.8 but were deemed less likely as they are located on the other side of these regulator and transport genes to the biosynthetic machinery.

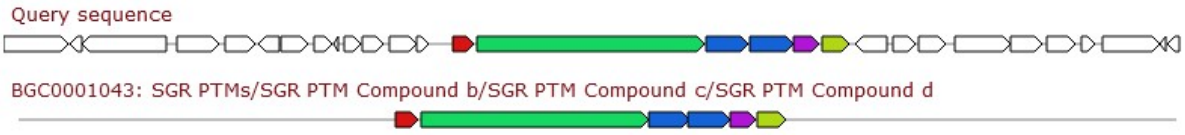


Figure 15 - Region 1.8 of *Streptomyces* B2 Predicted by antiSMASH (top) and Compared to the PTM b/c/d smBGC (bottom). All of the biosynthetic machinery encoding PTM b/c/d was predicted to be encoded within Region 1.8, highlighted by the coloured genes. Red genes are those with an unknown function, green encoding an NRPS, blue and purple tailoring enzymes and yellow cytochrome p450 enzymes. Where a gene has no colour, it was deemed to not have high homology to any gene in the other sequence.

Another example of a manual review is that of Region 23 of Amycolatopsis FG22, which was predicted to have a 66% similarity to the smBGC encoding the antifungal agent butyrolactol A previously identified in the genome of *Streptomyces sp. NBRC 110030* (Komaki *et al.*, 2015). Upon manual review, it was decided that it was in fact unlikely that butyrolactol A is encoded by Region 23. As shown in Figure 16, there were several gene in Region 23 that did not have significant homology to those in the butyrolactol A smBGC, such as *DJ578_31225*, which was predicted to encode an HNH endonuclease. HNH endonucleases cleave phosphodiester bonds, in particular – although not exclusively – during DNA repair and restriction of viral DNA (Keeble, Maté and Kleanthous, 2005). Additionally, Region 23 lacked a propionyl-CoA carboxylase that is found in the butyrolactol A PKS machinery. This may have a significant impact on the core structure on the molecule produced. Further, Region 23 lacked an oxidoreductase-encoding gene, which is found adjacent to the PKS machinery in the butyrolactol A and thus may influence the core structure of the produced compound. The butyrolactol A smBGC also contains a gene encoding a protein with unknown function at its peripheral. Whilst Region 23 does not contain a gene with a high similarity to this unknown gene, it cannot be ruled out that a different gene in a similar loci could convey that activity. Finally, Region 23 lacked a regulator and transporter genes in similar loci to those in the butyrolactol A smBGC, with these gene being found downstream of the biosynthetic machinery in Region 23 but spread throughout the butyrolactol A smBGC. This would not conclusively show that these two sequences do not encode the same or similar products, but when combined with the other observations made here, it was decided that it was unlikely that Region 23 encoded butyrolactol A.

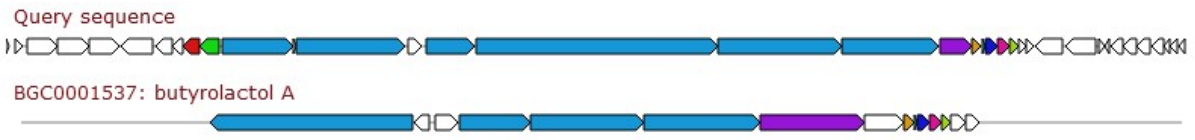


Figure 16 - Region 23 of *Amycolatopsis* FG22 Predicted by antiSMASH (top) and Compared to the butyrolactol A smBGC (bottom). It was deemed that several gene important to the biosynthesis of butyrolactol A were missing from Region 23 and that there were several other biosynthetic gene, which encoded proteins with different functions to those found in the butyrolactol A smBGC. Here, light blue and purple genes encode a PKS module, red a ligase, green an oxidase, orange and dark blue a dehydrogenase, magenta a phosphatase and lime green a hydrolase. Where a gene has no colour, it was deemed to not have high homology to any gene in the other sequence.

The largest genome was *Amycolatopsis* FG22 (10.22Mbp); the smallest was *Agrococcus* A6 (3.12Mbp). The *Streptomyces* A7 genome contained the highest number of smBGCs (40), whilst *Agrococcus* A6 contained the least (3). Of the total 404 smBGCs identified, 89 (22.0%) were determined to encode PKS, 24 (5.9%) for RiPPs, 83 (20.5%) for NRPS, 47 (11.6%) encoded for terpenes, and 28 (6.9%) were hybrid BGCs, made up of both PKS and NRPS. The remaining 133 (32.9%) smBGCs did not fit in these categories, or the BGC had so little resemblance to previously discovered smBGCs that it was unclear what biosynthetic machinery was encoded.

Of the BGCs identified, 88 (21.8%) had a high level of homology to previously discovered BGCs. High homology was defined as >80% similarity to a known BGC, or a manual review determining similarity was present. This demonstrates the biosynthetic potential still left to discover in both *Streptomyces* and other rare actinomycetes from the fungus-growing ant colony. The *Streptomyces* strains had the highest proportion of smBGCs with a >80% homology to previously discovered smBGCs (35.3%). This was followed by *Amycolatopsis* (13.2%), *Pseudonocardia* (8.7%), *Jiangella* (8.3%) with *Agrococcus* A6 and *Tsukamurella* E7 both having no smBGCs predicted to have high homology to previously discovered clusters.

Table 11 - Summary of antiSMASH Analysis of BGCs Present in Actinomycete Strains. High homology refers to an antiSMASH similarity score of 80% or higher, or a high homology found upon manual review. The green/yellow colour scaling is used in the *Total BGCs* column to highlight the relative number of BGCs each strain was predicted to contain, with green colouring indicating a larger number. The same green/yellow colouring is used in the *smBGCs/Mbp* and *BGCs with High Homology to Known Cluster(s)* columns. For the remaining columns, a heat map for using the yellow/green colouring highlights where strains encode a relatively high number of that type of BGC.

Strain	Genome Size (Mbp)	Total BGCs	smBGCs/Mbp	PKS-encoding BGCs	RiPP-encoding BGCs	NRPS-encoding BGCs	Terpene-Encoding BGCs	Hybrid BGCs	Other BGCs	BGCs with High Homology to Known Cluster(s)
Pseudonocardia UM4	*6.36*	14	2.20	1	0	3	1	3	6	2
Pseudonocardia UM14	*6.55*	19	2.90	4	1	4	2	1	7	1
Pseudonocardia UM9	*9.83*	16	1.63	2	0	2	1	3	8	1
Pseudonocardia P1	*6.81*	20	2.94	3	0	7	2	2	6	2
Streptomyces KY2	*8.58*	32	3.73	5	1	13	2	2	9	5
Streptomyces FG4	*6.72*	24	3.57	8	1	5	3	0	7	7
Streptomyces B2	*7.10*	19	2.68	1	1	6	3	2	6	8
Streptomyces FG7	*6.18*	23	3.72	6	1	6	3	0	7	7
Streptomyces A7	*8.36*	40	4.78	11	4	8	6	1	10	12
Streptomyces KY4	*9.03*	28	3.10	8	3	3	4	2	8	11
Streptomyces FG1	*8.02*	35	4.36	10	4	7	4	1	9	12
Streptomyces KY1	*6.96*	19	2.73	5	1	2	3	3	5	9
Amycolatopsis UM15	*10.22*	33	3.23	6	1	4	5	5	12	3
Amycolatopsis FG22	*9.00*	35	3.89	11	1	6	3	1	13	6
Agrococcus A6	3.12	3	0.96	1	0	0	0	0	2	0
Tsukamurella FG11	4.63	14	3.02	1	0	3	3	1	6	1
Tsukamurella E7	5.16	18	3.49	3	3	3	2	0	7	0

Jiangella S1	*7.35*	12	1.63	3	2	1	0	1	5	1
Total	129.98	404	N/A	89	24	83	47	28	133	88
Average	7.22	22.44	3.03	4.94	1.33	4.61	2.61	1.56	7.39	4.89

On average, the *Streptomyces* strains analysed contained 25.2 smBGCs each. This is less than the 36.5-40.0 seen in the previously studied *Streptomyces* strains (Belknap *et al.*, 2020; Lee *et al.*, 2021). The average genomes size of the *Streptomyces* strains in this study was 7.62Mbp, with *Streptomyces* KY4 having the largest (9.03Mbp) and *Streptomyces* FG7 the smallest (6.18Mbp). These genome sizes are typical of previously isolated *Streptomyces* strains, which have been reported to have a genome 6-12Mbp in length (Kim *et al.*, 2015). Despite having the largest genomes among the *Streptomyces* species reported here, *Streptomyces* KY4 only contains 32 antiSMASH predicted smBGCs, less than both *Streptomyces* A7 (40smBGCs) and FG1 (35smBGCs), both of which had smaller genomes (8.36Mbp and 8.02Mbp). Despite this, both *Streptomyces* KY1 and B2 had fewer smBGCs per Mbp (2.73smBGCs/Mbp, 2.68smBGCs/Mbp) than *Streptomyces* KY4 (3.1smBGCs/Mbp). *Streptomyces* A7 was the strain with the highest number of smBGCs for its genome size (4.78smBGCs/Mbp) for any of the strains analysed. The *Streptomyces* strains had on average a higher density of smBGCs (3.58smBGC/Mbp) than any other species in this study (*Pseudonocardia*, 2.42 smBGC/Mbp; *Amycolatopsis*, 3.56smBGC/Mbp; *Agrococcus,* 0.96smBGC/Mbp; *Tsukamurella* 3.26smBGC/Mbp; *Jiangella*, 1.63smBGC/Mbp). This could be partly due to more known about the smBGCs present in *Streptomyces* compared to the rare actinomycetes, causing a bias within antiSMASH towards finding smBGCs in these strains. This would lead to less smBGCs being detected by antiSMASH and thus also not noticed during the manual review of the antiSMASH outputs.

Pseudonocardia UM4, UM14, UM9 and P1 possessed an average of 17.25 smBGCs with an average genome length of 7.39Mbp. This is a typical genome size for *Pseudonocardia* species, which previously have been found to have a genome size of 5.05 to 10.33Mbp (Holmes *et al.*, 2016; Nouioui *et al.*, 2018; Safaei *et al.*, 2021). *Amycolatopsis* FG22 and UM15 possessed 35 and 33 smBGCs, respectively, more than *Streptomyces* FG4, B2, FG7, KY1, KY2, and KY4. This makes *Amycolatopsis* FG22 the third most talented strain studied after *Streptomyces* A7 and FG1, with *Amycolatopsis* UM15 fourth. This is slightly higher but comparable to previously discovered *Amycolatopsis* species, which possess an average of 29 smBGCs. Most *Amycolatopsis* strains have a genome size of 2-10Mbp, although one strain with a genome over 13Mbp in size has been isolated (Adamek *et al.*, 2018). With genomes 9.00 and 10.22Mbp respectively, *Amycolatopsis* FG22 and UM15 are on the large end of historically

observed genome size. *Agrococcus* A6 contained three smBGCs and had a genome size of 3.12Mbp. This is similar to previously studied *Agrococcus* species isolated from multiple environments, distinct from the insect microbiome, but the sample reported here did not contain any mobile elements, unlike some previously reported strains (Nouioui *et al.*, 2018; White *et al.*, 2018; Boxberger *et al.*, 2021; Soldatou *et al.*, 2021). *Tsukamurella* FG11 and E7 possessed 14 and 18 smBGCs, with a genome size of 4.63Mbp and 5.16Mbp respectively. Previously reported *Tsukamurella* strains have been found to have genomes of 4.5-5.2Mbp in size, meaning FG11 and E7 are typical in this regard, despite many of these previously reported strains being clinical isolates causing opportunistic infections in nosocomial settings (Christine Munk et al., 2011; Teng et al., 2016, 2020; Chiciudean et al., 2018; Nouioui et al., 2018). The genome sequence for *Jiangella* S1 showed a genome size of 7.35Mbp and 12 smBGCs. This is slightly larger than previously reported strains, with genomes ranging from 5.5 to 7.3Mbp in size (Jiao et al., 2017; Nouioui et al., 2018).

3.3.1 AntiSMASH Analysis of *Pseudonocardia* UM4

Pseudonocardia UM4 encoded 14 smBGCs, as shown in Appendix 7.1.1. One of these (Region 1.10) was predicted to be a PKS BGC encoding for the nystatin P1-like polyene, commonly observed in the Ps1-phenotype *Pseudonocardia octospinosus* species. Additionally, Region 1.3 was predicted to encode for ectoine, a highly conserved osmolyte that helps protect against high concentrations of salt and temperature. No other clusters demonstrated a high similarity to known clusters. Although three (Regions 1.7, 1.9 and 2.1) smBGCs were predicted by antiSMASH to encode NRPS products, one (Region 1.6) a terpene and three (Regions 1.1, 1.8 and 1.11) to be hybrid NRP/PKS clusters, the low similarities to known clusters means it is difficult to confirm this. Additionally, many of the BGCs identified by antiSMASH were relatively small, as low as 8239bp for Region 1.5, which contained the gene *DIT1507035*, predicted to encode a major facilitator superfamily protein, as well as *DBP_07045*, predicted to encode for a magnesium chelatase-like protein (Uniprot, 2020, 2021). Given that both these genes, and their encoded proteins, are predictions made computationally from metagenomic analysis, it is unclear how much can be concluded from this and if antiSMASH has assigned more smBGCs to *Pseudonocardia* UM4 than it contains.

The antiSMASH analysis of *Pseudonocardia* UM4 was compared with the biological activity observed in Section 3.2 and presented in Table 12. In addition to the 35 media all strains were grown on, *Pseudonocardia* UM4 was cultured upon SFM supplemented with 40g/L of glucose and SFM and SM12 supplemented with 40g/L of sucrose. This was performed after activity on FML was observed, which exerts high levels of osmotic pressure, to determine whether osmotic stress alone elicited the observed bioactivity. Osmotic pressure did stimulate the production of antimicrobials on SFM supplemented with 40g/L of sucrose, but in no other high osmotic pressure condition tested. This suggests that while osmotic pressure may contribute to the regulation of antimicrobials in *Pseudonocardia* UM4, it is not the only condition contributing to their regulation.

Table 12 - Bioassay Results of *Pseudonocardia* UM4 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Medium	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	S	S	N	N	P	P
MYM	N	N	N	N	N	N
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N
SM5	N	N	N	N	N	N
SM6	P	S	N	N	N	N
SM7	N	N	N	N	N	N
SM12	N	N	P	P	N	N
SM14	N	N	N	N	P	N
SM15	F	F	F	F	F	F
SM18	N	N	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	P	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	N	N	N
SM32	N	N	N	N	N	N
minNAG	N	N	N	N	N	N
SFM + 40g/L Glucose	N	N	N	N	P	N
SFM + 40g/L Sucrose	P	P	P	P	S	S
SM12 + 40g/L Sucrose	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	N	N
YP/C	N	N	N	N	N	N
ISP2	P	P	S	S	N	N
ISP4	N	N	N	N	N	N

SFM+CasAA	N	N	N	N	N	N

The observed nystatin P1-like polyene (Region 1.10) is the likely cause for *Pseudonocardia* UM4's ability to inhibit *C. albicans* on FML and SFM+40g/L sucrose. However, the antibacterial activity observed on FML, SM6, SM12, SN20, SFM+40g/L sucrose and ISP2 cannot be easily explained by the antiSMASH analysis of the *Pseudonocardia* UM4 genome. Given how little information is available for many of the predicted smBGCs, it is not possible to assign antibacterial activity to any individual smBGC. *Pseudonocardia* strains are poorly genetically tractable, so disrupting multiple smBGCs would be very challenging.

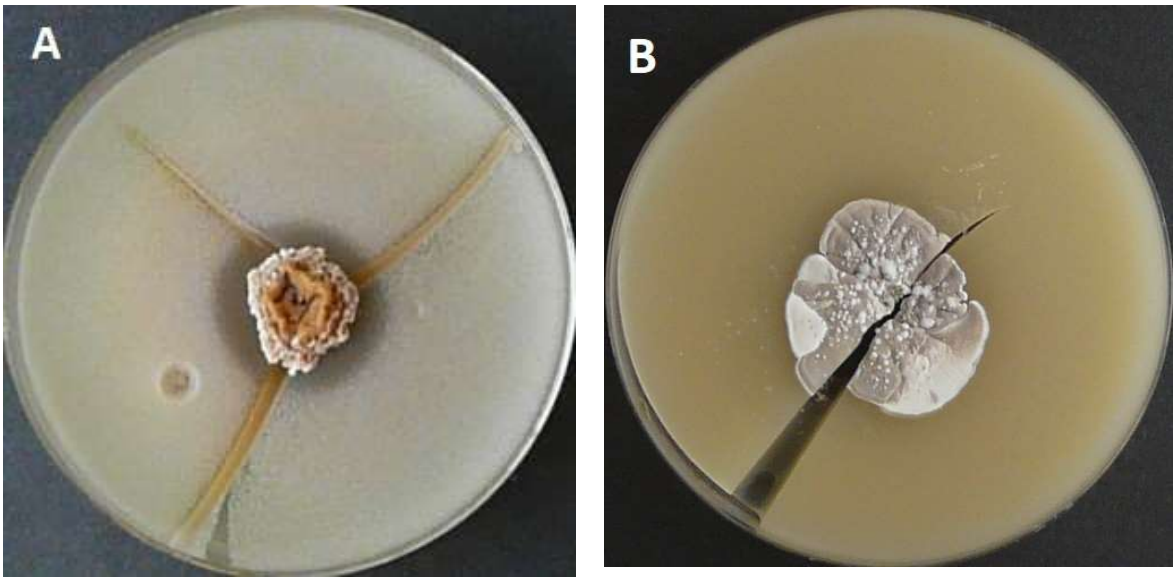


Figure 17 - Bioassays of *Pseudonocardia* UM4 when challenged by *C. albicans* on A - FML and B – SFM+Sucrose. These were assigned 'P' and 'S' respectively.

3.3.2 AntiSMASH Analysis of *Pseudonocardia* UM14

AntiSMASH analysis of *Pseudonocardia* UM14, presented in Appendix 7.2.2, identified 19 smBGCs. There is no nystatin-P1-like cluster, consistent with previous observations that *Pseudonocardia* isolated from lower attines do not encode for polyene antifungals. The *Pseudonocardia* UM14 genome was predicted to encode 4 PKS BGCs (Region 1, 5, 6 and 15), 1 RiPP BGC (Region 8), 4 NRPS encoding BGCs (Regions 4, 9, 11 and 16), 2 BGCs encoding for a terpene (Regions 3 and 19), and no NRPS/PKS hybrid BGCs. These smBGCs did not correspond with the smBGCs previously believed to be characteristic of the Ps2 phylotype - a bacteriocin cluster, an NRPS cluster, a genome island containing T1PKS and NRPS genes, a lassopeptide cluster, and a terpene cluster similar to the brasilicardin cluster (Holmes *et al.*,

2016). The antiSMASH analysis revealed that Region 13 had a 100% similarity with the ecotine-encoding BGC. Further, antiSMASH reported Region 4 had a 90% similarity to the coelibactin-encoding BGC in *S. coelicolor* A3(2); however, upon manual review, it was deemed unlikely that the actual similarity is this high (Bentley *et al.*, 2002). As seen in Figure 18, Region 4 in *Pseudonocardia* UM14 lacks *sco*7691, predicted to encode for an isochorismate synthase. Additionally, the *sco*7689 and *sco*7690 genes, both predicted to encode for adenosine triphosphate (ATP)-binding cassette (ABC) transporters, are geographically removed from the bulk of the genes identified as similar to those in the coelibactin BGC. To compound this, the predicted cytochrome P450 gene, assigned *DMP22_02960* by antiSMASH, encoded within Region 4 of *Pseudonocardia* UM14, only has a 57% identity to *sco*7686, the cytochrome P450 gene present in the coelibactin BGC. Additionally, this gene is encoded 5` to 3` in Region 4, rather than the 3` to 5` direction of sco7686 in the coelibactin BGC. This suggests that the similarity between Region 4 and the coelibactin smBGC is likely lower than the 90% predicted by antiSMASH. There is a chance that Region 4 does not encode a compound similar in structure or purpose to coelibactin.

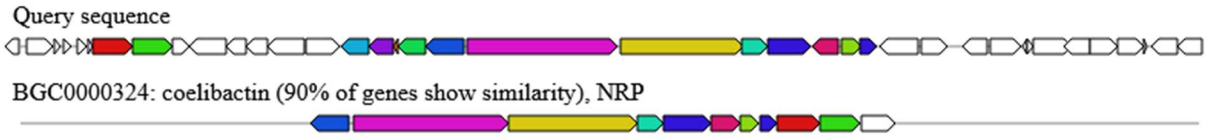


Figure 18 - Comparison of Region 4 in *Pseudonocardia* UM4 to *BGC0000324;* Encoding coelibactin in *Streptomyces coelicolor* A3(2) (Bentley *et al.*, 2002). Region 4 lacks *sco7691* – the uncoloured gene in the example coelibactin smBGC (below). The gene *sco7691* encodes isochorismate synthase, an isomerase enzyme that transfers the hydroxy group of chorismate between carbon atoms.

The antimicrobial activity of *Pseudonocardia* UM14, presented in Table 13, was compared to the antiSMASH analysis. As shown in Figure 19, antifungal activity was observed only on FML, and antibacterial activity against *B. subtilis* was only observed on FML and SM32. *Pseudonocardia* UM14 only sporadically grew on SM30 and SM32 and was one of 11 strains that consistently failed to grow on SM15. Both SM30 and SM32 are highly acidic media – both being adjusted to pH4.5 with HCl, which may explain the difficulty encountered by *Pseudonocardia* UM14. This does not explain why no growth was observed on SM15, which, at pH6.5, is less acidic than SM19 (pH6.0) and SM25 (pH6.3), upon which *Pseudonocardia* UM14 did grow.

Table 13 - Bioassay Results of *Pseudonocardia* UM14 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Medium	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	S	S	N	N	P	P
MYM	N	N	N	N	N	N
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N
SM5	N	N	N	N	N	N
SM6	N	N	N	N	N	N
SM7	N	N	N	N	N	N
SM12	N	N	N	N	N	N
SM14	N	N	N	N	N	N
SM15	F	F	F	F	F	F
SM18	N	N	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	N	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	F	N	F
SM32	S	F	N	F	N	N
minNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	N	N
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	S	S

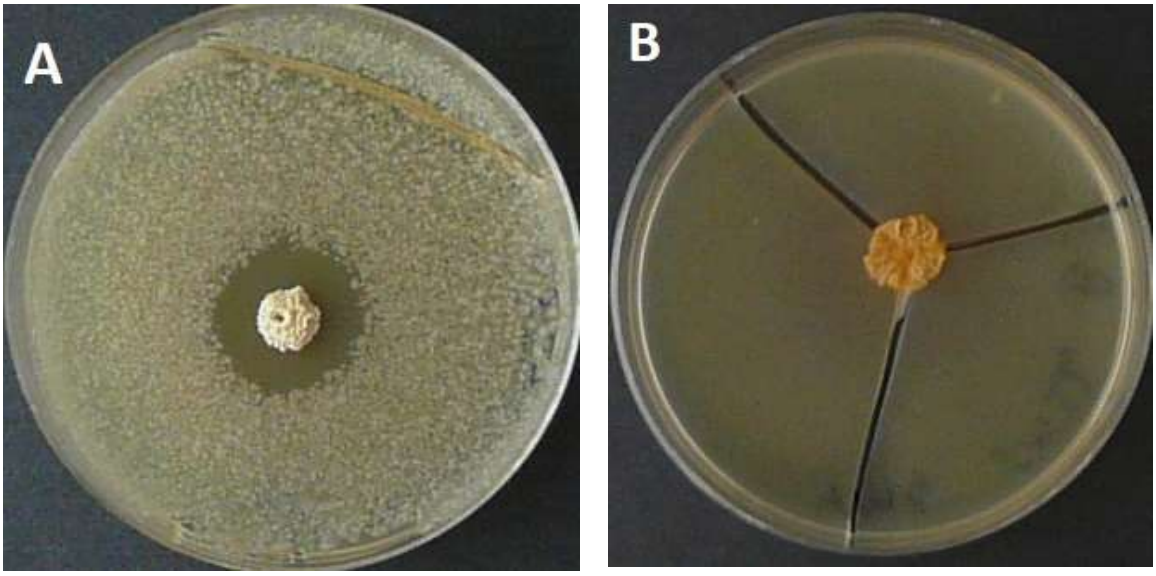


Figure 19 – Bioassays of *Pseudonocardia* UM4 on A – FML against *C. albicans* and B – SM32 against *B. subtilis.* These were assigned 'P' and 'N' respectively.

3.3.3 AntiSMASH Analysis of *Pseudonocardia* UM9

Appendix 7.2.3 presents the antiSMASH analysis of the *Pseudonocardia* UM9 genome. This analysis revealed 16 smBGCs. Only one of these, Region 2.5, had a high similarity to a previously known cluster, which had a 100% similarity to the ectoine BGC. The region with the next highest similarity to a known smBGC, Region 2.11, was predicted to have a 60% similarity to the smBGC for the siderophore scabichelin, lacking the *scab85481*, *scab85491* and *scab85501* genes involved in the transport of siderophores across the cell membrane, as seen in Figure 20. Despite being closely related to *P. echinatior* – of the Ps2 phenotype, *Pseudonocardia* UM9 did not contain the five genomic markers believed to be characteristic of the *P. echinatior* species nor the nystatin P1-like antifungal typical of higher-attine-associated *Pseudonocardia octospinosus* Ps1 species. This is expected, given that *Pseudonocardia* UM9 was isolated from a *Mycocepurus smithii* nest, a member of the lower attine (Schultz and Brady, 2008). AntiSMASH did, however, predict two PKS BGCs (Regions 2.7 and 2.8), two NRPS BGCs (Regions 2.9 and 2.11), one terpene-encoding BGC (Region 2.12) and two NRPS/PKS hybrid BGCs (Regions 2.1 and 2.2).

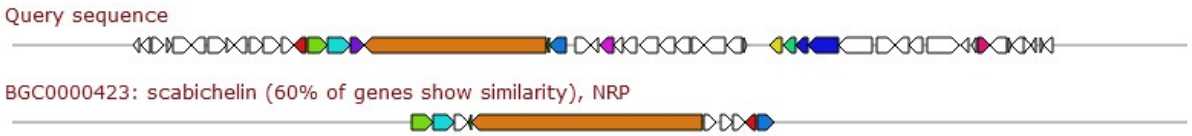


Figure 20 - Comparison of Region 2.11 in *Pseudonocardia* UM9 to *BGC0000423*; Encoding Scabichelin in *S. scabiei* 87.22 (Kodani *et al.*, 2013). Region 2.11 lacks *scab85481*, *scab85491* and *scab85501* gene encoding transporters, left colourless in the scabichelin smBGC (below).

None of the smBGCs identified by antiSMASH can explain the antimicrobial activity of *Pseudonocardia* UM9, as shown in Table 14. SFMNAG, SFMSB and GYM elicited antifungal activity, whilst antibacterial activity against the Gram-positive *B. subtilis* was elicited by SFMNAG, minNAG, SM3, 6 and 20. *Pseudonocardia* UM9 was one of the 11 strains that failed to grow on SM15.

Table 14 - Bioassay Results of *Pseudonocardia* UM9 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Medium	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	S	S	N	N	S	S
SFMSB	N	N	N	N	S	S
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	N	N	N	N	N	N
MYM	N	N	N	N	N	N
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	N	S
Minimal	N	N	N	N	N	N
SM3	P	P	N	N	N	N
SM5	N	N	N	N	N	N
SM6	S	S	N	N	N	N
SM7	N	N	N	N	N	N
SM12	N	N	N	N	N	N
SM14	N	N	N	N	N	N
SM15	F	F	F	F	F	F
SM18	N	N	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	S	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	N	N	N
SM32	N	N	N	N	N	N
minNAG	S	S	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	N	N
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.4 AntiSMASH Analysis of *Pseudonocardia* P1

AntiSMASH analysis of *Pseudonocardia* P1 revealed 20 smBGCs, as shown in Appendix 7.2.4. Region 1.1 had a 100% similarity for the nystatin-like polyene common to other Ps1-type *P. octospinosus* species, whilst Region 1.11 encoded ectoine. No other region had a high similarity to known smBGCs. Three (Regions 1.1, 1.19 and 2.1) of the smBGCs were predicted to encode for PKS BGCs and seven (Regions 1.2, 1.7, 1.8, 1.9, 1.12, 1.14 and 1.18) for NRPS. Regions 1.5 and 1.9 were predicted to encode a terpene, with two Regions (1.3 and 1.4) predicted to encode hybrid NRPS/PKS BGCs. The remaining six smBGCs were deemed 'other.' *Pseudonocardia* P1 demonstrated the ability to inhibit *C. albicans* on five different media; SFMNAG, YP, YPD, SM7 and SM12 -

Table 15. Activity against *B. subtilis* was observed on SM14 and SM30, whilst *E. coli* activity was only observed on SM18. The nystatin-like polyene smBGC likely explains the antifungal activity of *Pseudonocardia* P1, although it does not explain the antibacterial activity observed. The disconnected nature of Gram-positive and -negative activity suggests two separate compounds are responsible for the activity, although they proved hard to illicit. *Pseudonocardia* P1 failed to grow on SM15.

Table 15 - Bioassay Results of *Pseudonocardia* P1 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Medium	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	S	S
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	S	S
YPD	N	N	N	N	S	S
SPY	N	N	N	N	N	N
FML	N	N	N	N	N	N
MYM	N	N	N	N	N	N
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N
SM5	N	N	N	N	N	N

SM6	N	N	N	N	N	N
SM7	N	N	N	N	S	S
SM12	N	N	N	N	S	S
SM14	S	S	N	N	N	N
SM15	F	F	F	F	F	F
SM18	N	N	S	S	N	N
SM19	N	N	N	N	N	N
SM20	N	N	N	N	N	N
SM25	N	N	N	N	N	N
SM30	P	P	N	N	N	N
SM32	N	N	N	N	N	N
minNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	N	N
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.5 AntiSMASH Analysis of *Streptomyces* KY2

The antiSMASH analysis of *Streptomyces* KY2 revealed 32 smBGCs, of which five (Regions 4, 22, 23, 25 and 29) have high homology to known clusters. This is presented in Appendix 7.2.5. Region 4 is predicted to encode actinonin, a peptide deformylase inhibitor that functions as a broad-spectrum bacteriostatic agent and is also being investigated as an anti-tumour agent, potentially targeting these same peptide deformylases in human mitochondria (Dawn Z. Chen *et al.*, 2000; Hu *et al.*, 2020). Regions 22 and 23 encoded for ectoine and the siderophore desferrioxamine E respectively. Region 25 had a 100% similarity to an smBGC encoding for naseseazine C, an anti-plasmodial diketopiperazine initially isolated from a *Streptomyces* species derived from marine sediment in Australia (Buedenbender *et al.*, 2016).

Region 16 was predicted to possess 87% similarity to the puromycin encoding BGC in *S. alboniger.* As shown in Figure 21, puromycin is a structural analogue of tyrosyl-tRNA, possessing a peptide bond in place on an ester bond. This allows puromycin to enter the ribosomal A-site, and the free amino group can accept an incoming polypeptide chain at the P-site. However, the ester bond is unable to be cleaved by incoming tRNA molecules, leading to premature termination in translation and the disassembly of the 80S ribosome and the

exhibited antibacterial activity. The lack of similarity between the puromycin smBGC and Region 16 in *Streptomyces* KY2 is due to the lack of *orfA*, a gene deemed not part of the puromycin BGC and the purpose of which is undetermined (Tercero *et al.*, 1996; Aviner, 2020).

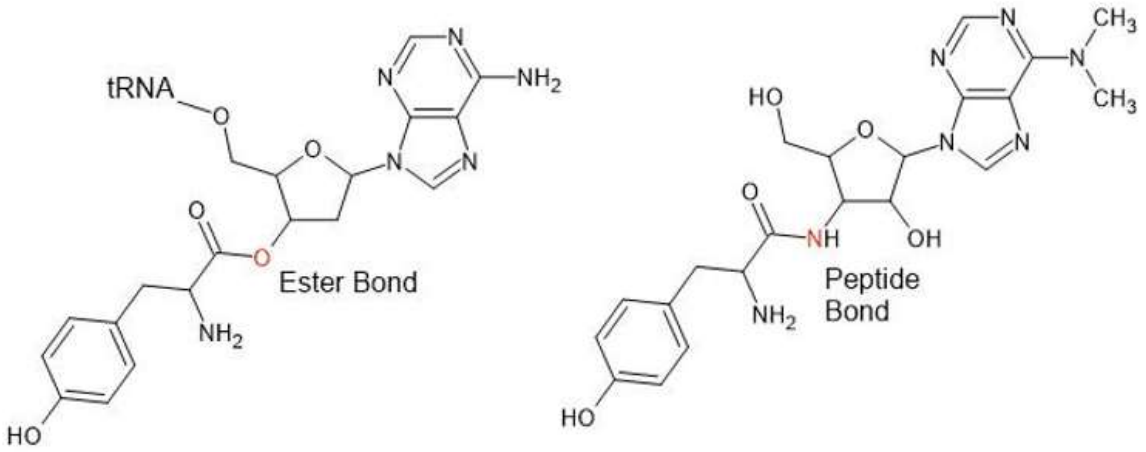


Figure 21 - The Structure of Tyrosyl-tRNA (left) and Puromycin (right)

Region 29 was predicted to have a 76% similarity to candicidin, an antifungal agent shown in Figure 22. *Streptomyces* KY2 lacks *fscO*, encoding a flavin adenine dinucleotide-dependent monooxygenase tailoring enzyme, the exact purpose of which in candicidin biosynthesis is unknown. Generally, enzymes belonging to the flavin adenine dinucleotide-dependent monooxygenase family catalyse hydroxylation, epoxidation and halogenation reactions, leading to speculation that FscO is responsible for C-9 hydroxylation, C10 hydroxylation or the conversion of the C-18 hydroxyl to an aldehyde. All these proposals are flawed, however, being unprecedented (C-9 hydroxylation), there being no evidence of the edit in the final structure (C-10 hydroxylation) or requiring a dehydratase to be silent in order to leave an unreduced hydroxyl group on C-9, which would allow for the conversion of the C-18 hydroxyl to an aldehyde (C-18 conversion) (Chen *et al.*, 2003; Heine *et al.*, 2018). It seems likely that even without the FscO tailoring enzyme, the compound encoded by Region 29 would maintain anti-fungal activity as the bulk of the structure remains consistent with candicidin, suggesting the pharmacophore is intact. Further, *Streptomyces* KY2 lacks both *FscRIII* and *FscRIV*, encoding LuxR-family regulators, which are replaced by the hypothetical genes, assigned *dkb27_33380* and *dkb27_33385* by antiSMASH. These encode alternative LuxR-family regulators similar to those found in other *Streptomyces* species, as shown in

Table 16 and

Table 17 respectively. The two genes were compared using BlastP and found to have limited similarity, with a query cover of 23% and a percentage identity of 31.70%, suggesting these are two different regulatory regions. It seems unlikely that changing these two LuxR receptor sites would impact the biological activity of the compound produced. Thus, it was determined that Region 29 is most likely responsible for encoding the compound causing anti-fungal activity by *Streptomyces* KY2, as displayed in

Table **18**.

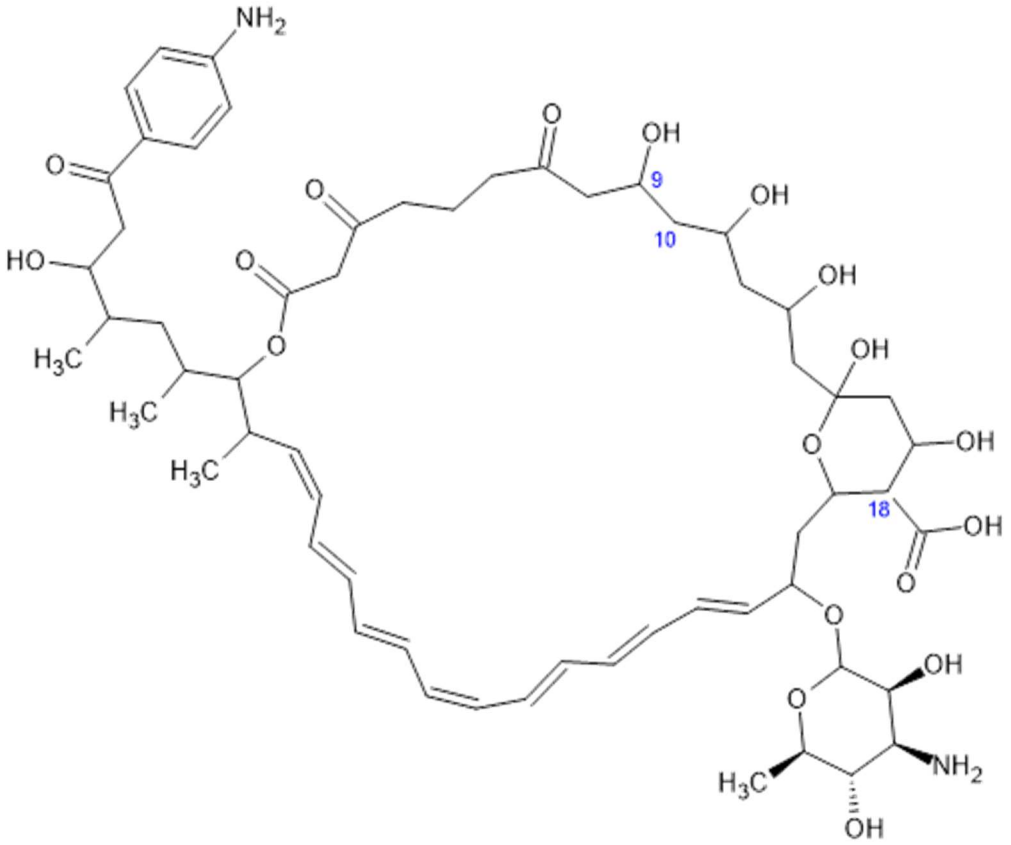


Figure 22 - The Structure of Candicidin, FscO is suspected of interacting at one of the labelled carbons – C-9 (hydroxylation), C-10 (hydroxylation), or C-18 (conversion of hydroxy to aldehyde, assuming the hydroxyl group at C-9 was left unreduced)

Table 16 – BlastP Analysis of *dkb27_33380*, demonstrating its high similarity to LuxR family regulators present in other *Streptomyces* species

Description	Scientific Name	Query Cover	E value	Identity (%)	Accession
LuxR family transcriptional regulator	*Streptomyces sp.* NRRL S-1868	100%	0	99.47	WP_037865933.1
LuxR family transcriptional regulator	*Streptomyces cacaoi*	100%	0	99.47	WP_086816520.1
LuxR family transcriptional regulator	*Streptomyces cacaoi*	100%	0	99.68	WP_149563409.1
LuxR family transcriptional regulator	*Streptomyces sp.* NHF165	100%	0	99.47	WP_159786833.1
LuxR family transcriptional regulator	*Streptomyces sp.* NRRL F-5053	100%	0	99.47	WP_037849011.1
AAA family ATPase	*Streptomyces xantholiticus*	98%	0	56.91	WP_189882689.1
LuxR family transcriptional regulator	*Streptomyces sp.* CoT10	99%	0	56.6	WP_225079179.1
LuxR family transcriptional regulator	*Streptomyces sp.* 6-11-2	99%	0	56.6	WP_141364258.1
LuxR family transcriptional regulator	*Streptomyces nodosus*	99%	0	55.45	WP_052453978.1
AAA family ATPase	*Streptomyces peucetius*	98%	0	55.73	WP_100109812.1

Table 17 - BlastP Analysis of *dkb27_33385*, demonstrating its high similarity to LuxR family regulators present in other *Streptomyces* species

Description	Scientific Name	Query Cover	E value	% identity	Accession
LuxR family transcriptional regulator	*Streptomyces cacaoi*	100%	0	99.68	WP_149563409.1
LuxR family transcriptional regulator	*Streptomyces sp.* NHF165	100%	0	99.47	WP_159786833.1
LuxR family transcriptional regulator	*Streptomyces sp.* NRRL S-1868	100%	0	99.47	WP_037865933.1
LuxR family transcriptional regulator	*Streptomyces cacaoi*	100%	0	99.47	WP_086816520.1
LuxR family transcriptional regulator	*Streptomyces sp.* NRRL F-5053	100%	0	99.47	WP_037849011.1
LuxR family transcriptional regulator	*Streptomyces sp.* CoT10	99%	0	59.15	WP_225079179.1
AAA family ATPase	*Streptomyces xantholiticus*	98%	0	58.41	WP_189882689.1
LuxR family transcriptional regulator	*Streptomyces sp.* 6-11-2	99%	0	59.15	WP_141364258.1
LuxR family transcriptional regulator	*Streptomyces sp.* CB01373	99%	0	58.09	PJM92793.1
LuxR family transcriptional regulator	*Streptomyces nodosus*	98%	0	57.05	WP_052453978.1

Despite there being a proposed smBGC encoding biosynthesis of the antifungal candicidin in *Streptomyces* KY2, *C. albicans* inhibition was only elicited on five growth media (SFM, YP, YPD, SM25 and GYM+2µg/mL streptomycin) whereas *B. subtilis* inhibition was elicited on 12 media (SFM, SFMNAG, SFMSB, YP, YPD, SPY, FML, YEME, GYM, SM18, SM20 and SM25) and *E. coli* on four (FML, Minimal, SM25 and GYM+2 µg/mL streptomycin). The activity observed against all three indicator strains can be predicted from the antiSMASH analysis – the *C. albicans* inhibition is likely due to candicidin (Region 29), whilst *B. subtilis* and *E. coli* could be inhibited by puromycin (Region 16). However, *B. subtilis* and *E. coli* inhibition was disassociated – *E. coli* inhibition was observed on Minimal and GYM+2 µg/mL streptomycin, but *B. subtilis* was not, whilst *B. subtilis* inhibition was observed on multiple media, including SM20 and YEME, where *E. coli* was not inhibited. This suggests that two different compounds are responsible for these activities.

Table 18 - Bioassay Results of *Streptomyces* KY2 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Medium	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	P	P	N	N	S	S
SFMNAG	P	P	N	N	N	N
SFMSB	P	P	N	N	N	N
YP	S	S	N	N	S	S
YPD	S	S	N	N	S	S
SPY	P	P	N	N	N	N
FML	P	P	S	S	N	N
MYM	N	N	N	N	N	N
YEME	S	S	N	N	N	N
IMA	N	N	N	N	N	N
GYM	P	P	N	N	N	N
Minimal	N	N	P	P	N	N
SM3	N	N	N	N	N	N
SM5	N	N	N	N	N	N
SM6	N	N	N	N	N	N
SM7	N	N	N	N	N	N
SM12	N	N	N	N	N	N
SM14	N	N	N	N	N	N
SM15	F	F	F	F	F	F
SM18	S	S	N	N	N	N
SM19	N	N	N	N	N	N
SM20	P	P	N	N	N	N
SM25	S	S	N	S	S	S
SM30	N	N	N	N	N	N
SM32	N	N	N	N	N	N
minNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	S	S	S	S
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N

SFM+CasAA	N	N	N	N	N	N

3.3.6 AntiSMASH Analysis of *Streptomyces* B2

Analysis of the *Streptomyces* B2 genome revealed 19 smBGCs, presented in Appendix 7.2.6. One of these encoded a PKS (Region 1.9), one RiPP (Region 1.1), six NRPS (Regions 1.3, 1.11, 1.13, 1.15, 1.16 and 1.17), three terpenes (Regions 1.6, 1.14 and 1.19), two hybrid PKS/NRPS (Regions 1.5 and 1.8) and six that were assigned as other (Regions 1.2, 1.4, 1.7, 1.10, 1.12 and 1.18). Nine of these had high homology to previously discovered BGCs. Region 1.1 had 100% homology to a BGC first identified in *S. griseus* encoding for the lanthipeptide AmfS, an extracellular regulatory molecule involved in regulating the aerial-mycelium formation and antibiotic production (Ueda et al., 2002). Regions 1.2 and 1.6 encoded the previously discussed ectoine and geosmin, respectively. Region 1.8 had 100% similarity to another *S. griseus* BGC, encoding a polycyclic tetramate macrolactam, a class of natural products with broad-spectrum antimicrobial activity commonly produced by *Streptomyces* species, as well as other bacteria. Polycyclic tetramate macrolactams can complex with a wide variety of metal ions, including copper, iron and nickel, which was once believed to relate to their antimicrobial activity, although this was subsequently disproven, and their mechanism of action remains unknown (Luo et al., 2013; Ding et al., 2021). Region 1.11 was predicted to have 100% similarity with the scabichelin-encoding *BGC0000423* from *S. scabiei* 87.22 (Kodani et al., 2013). Region 1.14 was determined by antiSMASH to have a 76% similarity to the hopene BGC from *S. coelicolor* A3(2) but lacked *sco6767*, encoding for a GcpE superfamily protein commonly found in terpene biosynthetic pathways (Hecht et al., 2001; Bentley et al., 2002). Given this difference, it is likely that Region 1.14 encodes a structural analogue of hopene. Region 1.16 was predicted by antiSMASH to have an 83% similarity to the BGC encoding the siderophore paenibactin produced by *Paenibacillus elgii* B69, lacking the paebacillibactin esterase *paeG* (Wen et al., 2011). However, it would appear more likely that Region 1.16 encodes for a different siderophore – bacillibactin from *B. velezensis* FZB42, the smBGC for which is highly similar to that for paenibactin but lacks *paeG* (Chen et al., 2009). Region 1.17 was predicted to encode coelibactin, a zincophore and regulator of antimicrobial production in *S. coelicolor* A3(2) (Bentley et al., 2002; Zhao et al., 2012). Region 1.19 was predicted to have an 85% similarity to the BGC encoding isorenieratene, a carotenoid class

compound produced by *S. griseus* NBRC 13350, likely a spore pigment and an anti-oxidant, although *Streptomyces* B2 lacks *crtT*, encoding a methyltransferase (Krügel et al., 1999).

As presented in

Table 19, *Streptomyces* B2 could not inhibit *E. coli* on any of the growth media tested; it could only inhibit *B. subtilis* on SM6, SM7, SM18 and MinNAG, and *C. albicans* on SM6. This is seen in Figure 23. This suggests that the antimicrobials produced by *Streptomyces* B2 are hard to elicit. Given the presence of a polycyclic tetramate macrolactam in the genome, this likely is the origin of this antimicrobial activity; the lack of activity against *E. coli* could be explained

either by the concentration being too low or because the specific polycyclic tetramate macrolactam produced by *Streptomyces* B2 is unable to inhibit Gram-negative bacteria, potentially due to an inability to penetrate the outer membrane.

Table 19 - Bioassay Results of *Streptomyces* B2 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Medium	Target Strain		
	B. subtilis	***E. coli***	***C. Albicans***

SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	N	N	N	N	N	N
MYM	N	N	N	N	N	N
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N
SM5	N	N	N	N	N	N
SM6	S	S	N	N	P	N
SM7	S	S	N	N	N	N
SM12	N	N	N	N	N	N
SM14	N	N	N	N	N	N
SM15	N	N	N	N	N	N
SM18	S	S	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	N	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	N	N	N
SM32	N	N	N	N	N	N
MinNAG	S	S	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	N	N
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

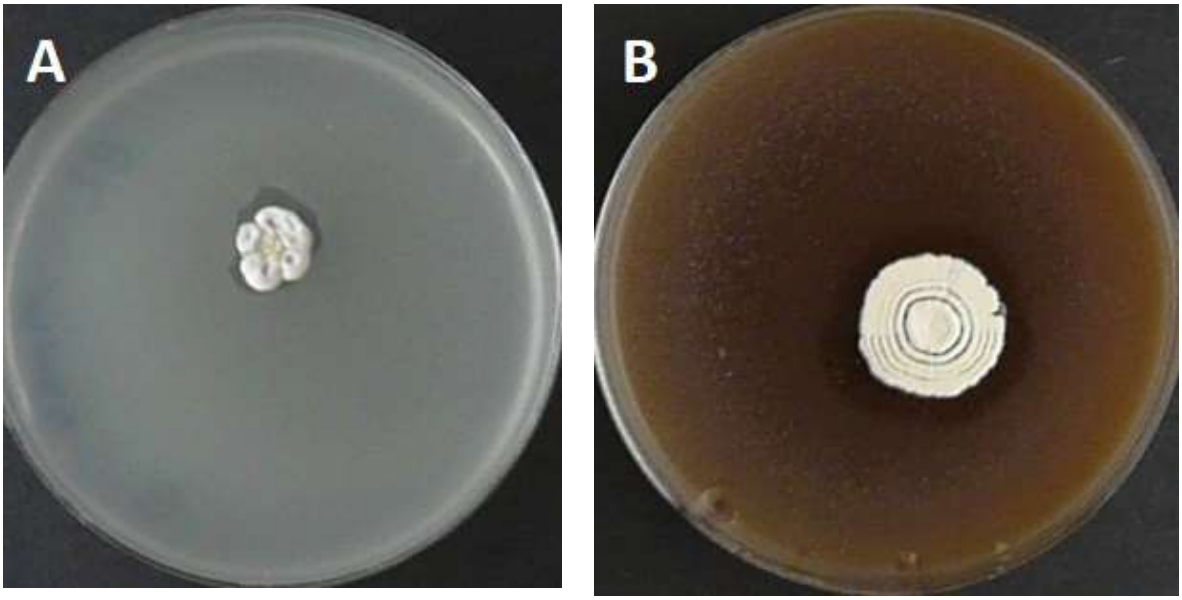


Figure 23 – Bioassays of *Streptomyces* B2 Against *B subtilis* on A – MinNAG and B – SM18. These were both assigned 'S'.

3.3.7 AntiSMASH Analysis of *Streptomyces* FG4

Analysis of the *Streptomyces* FG4 genome, presented in Appendix 7.2.7, revealed 24 smBGCs, eight of which encoded PKS (Regions 1.4, 1.5, 1.9, 1.11, 1.13, 1.17, 1.21 and 1.24), one RiPP (Region 1.23), five NRPS (Regions 1.1, 1.2, 1.14, 1.20 and 1.22), three terpene (Regions 1.10, 1.16 and 1.19) and seven others (Regions 1.3, 1.6, 1.7, 1.8, 1.9, 1.12, 1.15 and 1.18). Seven had high homology to previously discovered BGCs (Regions 1.2, 1.6, 1.8, 1.9, 1.10, 1.16, 1.18 and 1.19). Region 1.2 had a 100% similarity to the scabichelin BGC in *S. scabiei*. Region 1.6 had a 100% similarity to ectoine, whilst Region 1.8 had an 83% similarity to the BGC encoding the siderophore desferrioxamine, the main difference being the lack of *sco2780*, a gene encoding for a ferric-siderophore lipoprotein receptor. Region 1.9 also had an 83% similarity, but instead to a BGC encoding a spore pigment with an uncharacterised structure from *S. avermitilis* (Omura et al., 2001). *Streptomyces* FG4 lacks the regulators that flank the spore pigment BGC in *S. avermitilis*. Instead, the genes that have high homology to those found in the spore pigment are flanked by more biosynthetic machinery – a hydroxyurea phosphotransferase and a glycosyl transferase, as shown in Figure 24. The actual compound encoded by this BGC may have little relation to the spore pigment given the surrounding biosynthetic machinery, although further study would be required to determine this. Region 1.10 had 100% similarity to a BGC encoding the antibacterial agent albaflavenone in *S. coelicolor* A3(2). The albaflavenone BGC consists of two genes, *sco5223* encoding the

cytochrome P450 170A1 and *sco5222*, an epi-isozizaene synthase that results in repeated cyclisation reactions followed by several two- and three-step oxidation reactions (Zhao et al., 2008). A similar story presents itself for Region 1.16, predicted to encode for geosmin, a highly conserved odorant responsible for the characteristic smell of *Streptomyces* species, that of wet soil. In *Streptomyces* FG4, the predicted geosmin synthase is surrounded by other biosynthetic machinery, although in this case, these genes are unlikely to interact with the geosmin synthase as this is common with other *Streptomyces* strains (Jiang, He and Cane, 2007).

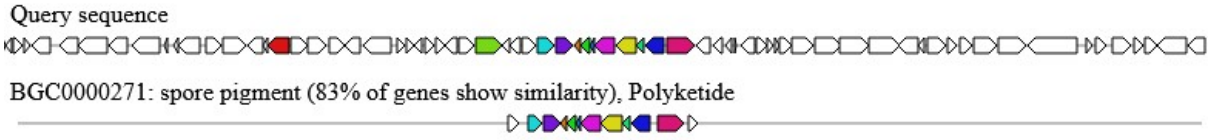


Figure 24 - Comparison of *Streptomyces* FG4 Region 1.9 with a BGC Encoding an Uncharacterised Spore Pigment in *S. avermitilis*. The two genes flanking the spore pigment encode regulator regions; however, the genes in Region 1.9 with high homology to the spore pigment BGC are flanked by more biosynthetic machinery.

Region 18 was predicted by antiSMASH to have a 61% similarity to the kanamycin BGC in *S. kanamyceticus* 12-6, an industrial strain modified from the wild type to enhance kanamycin biosynthesis. Region 18 contains a number of the genes encoding for the production of kanamycin, including the regulatory genes *dmg05_24885* and *di536_24905*, the membrane transporter encoding *G3545_24915* and the core biosynthetic machinery encoding genes such as *orf25-32*. However, *Streptomyces* FG4 lacks other biosynthetic genes, including *kanD3*, encoding a 3-hydroxyacyl-CoA dehydrogenase, and *kanM1*, encoding a glycosyltransferase (Kharel et al., 2004; Yanai, Murakami and Bibb, 2006). This BGC may be encoding a structural analogue of kanamycin. To test this, a kanamycin-resistant version of *E. coli* NCTC 12923 was generated by inserting a pET28a plasmid containing *kanR*, a gene encoding for an aminoglycoside 3'-phosphotransferase to phosphorylate kanamycin and prevent the antibiotic binding to 30S rRNA, as per Section 2.9 (McKay and Wright, 1996). Bioassays of *Streptomyces* FG4 against this kanamycin-resistant strain of *E. coli* were performed on SM6 and SM12. As shown in Figure 25, *Streptomyces* FG4 was equally able to inhibit the growth of wild-type *E. coli* NCTC 12923 and the pET28a-carrying kanamycin-resistant strain. This indicates that if *Streptomyces* FG4 produces a kanamycin analogue, it can evade modification of the aminoglycoside 3'-phosphotransferase encoded by *kanR* or is

modified in such a way that does not prevent binding to the 30S rRNA. Alternatively, a second antimicrobial may be produced, entirely unrelated to kanamycin, which is the cause of *E. coli* inhibition. Finally, Region 1.19 has 100% similarity to the hopene-encoding BGC in *S. coelicolor* A3(2), a membrane stabilising compound used as a precursor in a wide range of industrial processes (Bentley et al., 2002; Liu et al., 2020).

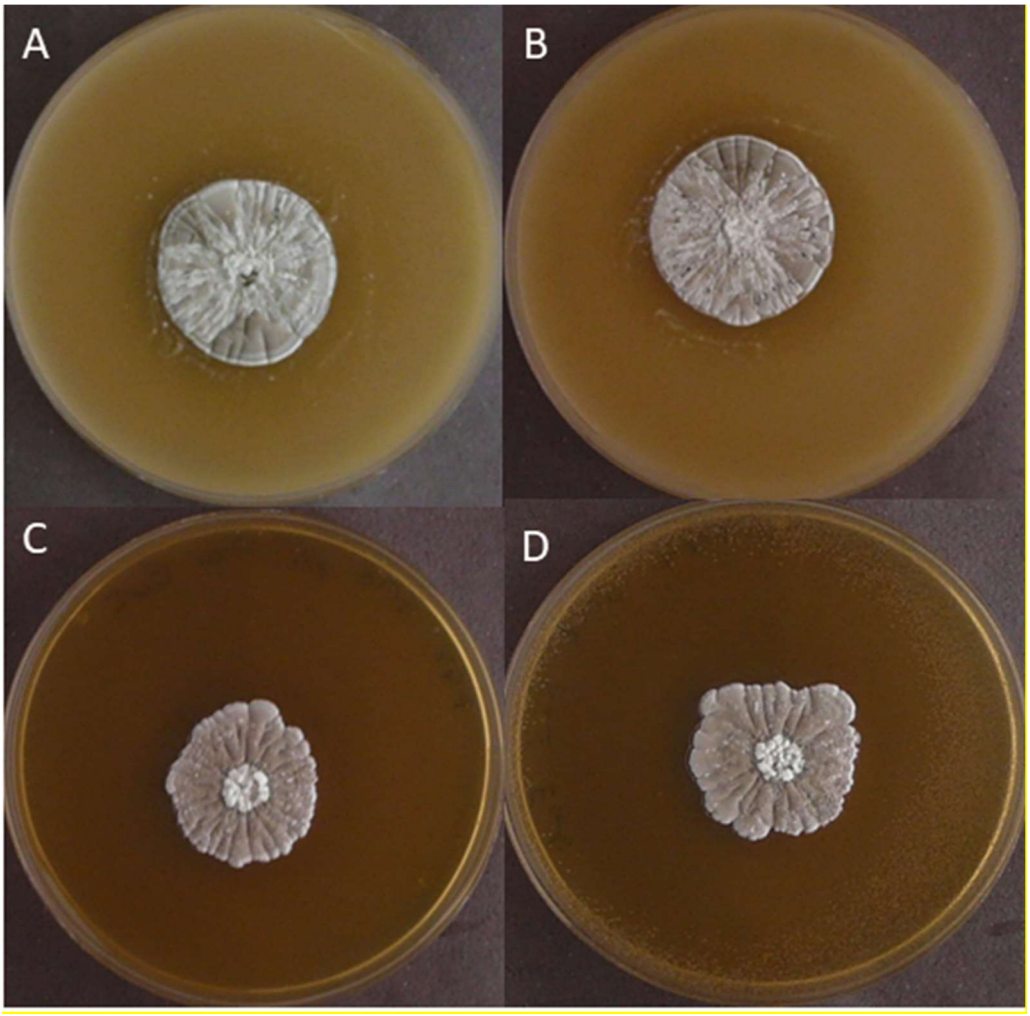


Figure 25 – *Streptomyces* FG4 when Challenged by; A: Wild-Type *E. coli* on SM12, B: pET28a *E. coli* on SM12, C: Wild-Type *E. coli* on SM6, D: pET28a *E. coli* on SM6. A similar sized zone of clearance can be seen when *Streptomyces* FG4 is challenged by either wild-type *E. coli* or pET28a *E. coli*.

The biological activity of *Streptomyces* FG4, presented in

Table 20, showed it could inhibit *B. subtilis*, *E. coli* and *C. albicans*. On minimal, SM12 and minNAG, *Streptomyces* FG4 was able to inhibit all three pathogens. In all instances of *E.* coli inhibition, *B. subtilis* inhibition was also observed, but the inverse was not necessarily true. It is unclear if two separate compounds are causing this or if *E. coli* processes a higher MIC for a single compound and only some media upregulate the encoding BGC sufficiently. Perhaps the former explanation is more plausible, as most compounds capable of inhibiting Gram-negative bacteria can also inhibit Gram-positive species. *C. albicans* activity was disconnected to both *B. subtilis* and *E. coli* activity, suggesting that a different compound provides the antifungal activity to antibacterial.

Table 20 - Bioassay Results of *Streptomyces* FG4 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Medium	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	P	P	N	N	S	S
SFMNAG	S	S	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	S	S	N	N	S	S
MYM	P	P	N	N	N	N
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	S	S
Minimal	S	S	P	P	S	S
SM3	P	P	N	N	N	N
SM5	N	N	N	N	N	N
SM6	P	P	P	P	N	N
SM7	S	S	N	N	N	N
SM12	S	S	P	P	S	N
SM14	N	N	N	N	N	N
SM15	S	S	N	N	N	N
SM18	N	N	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	N	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	N	N	N
SM32	N	N	N	N	N	N
minNAG	P	P	S	S	S	S
GYM + 10μg/mL streptomycin	S	S	P	P	N	N
GYM + 2μg/mL streptomycin	N	N	N	N	S	S
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.8 AntiSMASH Analysis of *Streptomyces* FG7

Streptomyces FG7 was determined to contain 23 smBGCs within its genome, six of which were determined to be PKS-encoding (Regions 1, 2, 4, 10, 20 and 22), one a RiPP (Region 21), six NRPS (Regions 3, 9, 13, 17, 19 and 23), three terpenes (Regions 11, 16 and 18) and seven other (Regions 5, 6, 7, 8, 12, 14 and 15) with no hybrid BGCs. Of these, seven had high homology to known smBGCs. This is presented in Appendix 7.2.8. Region 2 had 100% similarity to the alkyl resorcinol BGC in *S. griseus* NBRC 13350, a phenolic lipid that controls the rigidity of the cell membrane (Funabashi, Funa and Horinouchi, 2008). Region 5 had 100% similarity to ectoine, and Region 8 had 83% similarity to the siderophore desferrioxamine. Region 10 had an 83% similarity to an uncharacterised spore pigment produced by *S. avermitilis* (Omura et al., 2001). Region 11 was predicted to encode for albaflavenone - previously discussed in Section 3.3.7, whilst Region 16 had 100% similarity to the geosmin BGC from *S. coelicolor* A3(2) (Jiang, He and Cane, 2007). Region 18 had a 92% similarity to hopene, with *Streptomyces* FG7 lacking *sco6761*, which encodes for an uncharacterised protein in *S. coelicolor* A3(2) (Bentley et al., 2002).

As presented in

Table 21, *Streptomyces* FG7 failed to demonstrate activity against *E. coli* on any of the media tested and was able to inhibit *C. albicans* only on SM6. *B. subtilis* was inhibited on SM14, SM20 and YPC. This suggests that the albaflavenone-encoding BGC was unlikely to be elicited by the media tested or that this predicted smBGC did not encode albaflavenone nor for an alternative antimicrobial.

Table 21 - Bioassay Results of *Streptomyces* FG7 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Medium	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	N	N	N	N	N	N
MYM	N	N	N	N	N	N
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N
SM5	N	N	N	N	N	N
SM6	N	N	N	N	S	S
SM7	N	N	N	N	N	N
SM12	N	N	N	N	N	N
SM14	S	S	N	N	N	N
SM15	N	N	N	N	N	N
SM18	N	N	N	N	N	N
SM19	N	N	N	N	N	N

SM20	S	S	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	N	N	N
SM32	N	N	N	N	N	N
minNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	N	N
YP/C	S	S	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.9 AntiSMASH Analysis of *Streptomyces* A7

Streptomyces A7 was determined to be the most biosynthetically promising strain in this study, containing 40 smBGCs, although antiSMASH determined there to be 38. As discussed below, Region 32 was determined to be two smBGCs rather than the one predicted by antiSMASH and thus was split into Regions 32a and 32b. For the same reason, Region 33 was split into Regions 33a and 33b. Appendix 7.2.9 presents this updated view after a review of the antiSMASH data. Eleven of the encoding BGCs were predicted to encode PKS BGCs (Regions 1, 2, 7, 9, 17, 18, 20, 31, 33, 35 and 38), four RiPPs (Regions 12, 16, 25 and 36), six NRPS (Regions 8, 22, 23, 29, 32.2, 34 and 37), six terpenes (Regions 5, 15, 19, 24, 26 and 32.1), one hybrid NRPS/PKS (Region 30) and 10 other (Regions 3, 4, 6, 10, 11, 13, 14, 21, 27 and 28). Of these, 12 had high homology to previously discovered smBGCs; Region 1 has a 93% similarity to the lobophorin A smBGC from *Streptomyces sp.* FXJ7.023, a broad-spectrum antimicrobial that also has anti-tumour characteristics. *Streptomyces* A7 lacked the LuxR regulator and MATE efflux pump present in *Streptomyces sp.* FX18.023, which does not rule out Region 1 from encoding lobophorin A (Minghao et al., 2016). Region 3 had 100% similarity to the germicidin smBGC from *S. argillaceus* – an autoregulator of spore germination produced by spores during germination to inhibit the germination of nearby spores. At higher concentrations, germicidin can also inhibit the germination of cress seeds (Petersen et al., 1993; Becerril et al., 2018). Region 9 was predicted to have an 86% similarity to an smBGC from *Streptomyces sp.* TP-A0882 encodes buryrolactol, a γ-lactone containing antifungal agent capable of inhibiting *C. albicans* and condidium fungi, such as *Aspergillus fumigatus* (Kotake et al., 1992; Komaki et al., 2015). Region 10 had a 100% similarity to the ectoine BGC,

Region 13 83% to the desferrioxamine BGC, Region 19 100% to the antimicrobial albaflavenone BGC, and Region 26 100% to geosmin. All of these have previously been discussed.

Region 22 in *Streptomyces* A7 was predicted to have a 75% similarity to the friulimicin A/B/C/D encoding BGC in *Actinoplanes friuliensis*. Friulimicin is a lipopeptide antibiotic that targets the pentapeptide transferase enzyme utilised in bacterial cell wall synthesis. However, as shown in Figure 26, Region 22 has nine genes with low homology to their equivalents encoded in the friulimicin BGC – with six being at the 5` end of the smBGC. One of the genes at the 5` end of the BGC is *regE*, a TetR family regulator; the other is *mem2*, a drug resistance transporter. That these two genes have poor homology does not rule out the potential for Region 22 to encode friulimicin, although there are no similar genes in *Streptomyces* A7 at this loci, as drug efflux could be encoded elsewhere in the Region. Given that Region 22 has over 20 genes in addition to those in the friulimicin encoded at the 3` end of the Region, including a LuxR regulator and a major facilitator superfamily transporter, these genes may encode proteins providing similar functions to RegE and TetR in *Actinoplanes friuliensis*. It is, therefore, possible that Region 22 encodes either friulimicin A/B/C/D or a structural analogue of this family of compounds.

Figure 26 - AntiSMASH Predictions of Region 22

AntiSMASH predicted that Region 32 had a 92% similarity to the terpene BGC from *S. coelicolor* A3(2) encoding hopene, with *Streptomyces* A7 lacking the same *sco6761* gene that was absent in *Streptomyces* FG7. However, as shown in Figure 27, antiSMASH also predicted that a different section of Region 32 had 100% homology to a different smBGC – the coelibactin BGC from *S. coelicolor* A3(2). It was determined that Region 32 likely contained two smBGCs – labelled Region 32a for the section with a 92% similarity to the hopene BGC and Region 32b for the section with 100% similarity to the coelibactin BGC. It was predicted that 15 genes were present between the two smBGCs identified, including a gene assigned *D7X3032335* by antiSMASH, predicted to be a MarR-family regulator binding domain from 7,293,026bp to 7,293,560bp, a considerable distance from either predicted BGC - 6915bp

from Region 32a and 8303bp from Region 32b, suggesting that it does not regulate either of these nearby BGCs. It is unclear, however, if this forms a third smBGC in-between Regions 32a and b.

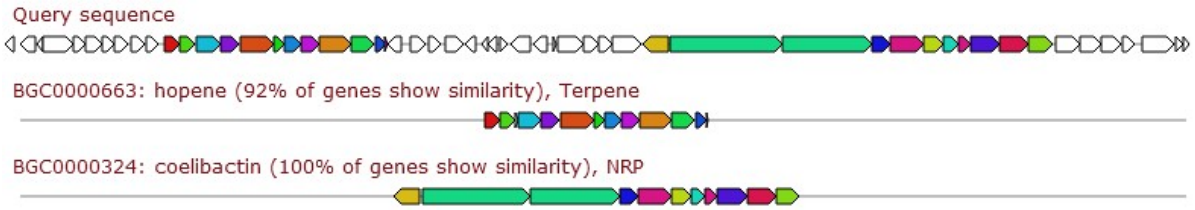


Figure 27 - AntiSMASH Prediction of Region 32. Upon manual review, it was determined that this region contained two smBGCs - one with a 92% similarity to the BGC encoding hopene and the other with 100% similarity to the coelibactin encoding BGC found in *S. coelicolor* A3(2). These were thus labelled as Regions 32a and 32b, respectively.

In addition, Region 33 was determined to contain two smBGCs labelled as Region 33a, from 7,376,378bp to 7,400,751bp, and Region 33b, from 7,421,725bp to 7,494,125bp, visualised in Figure 28. Region 33a was predicted to have a 78% similarity to mirubactin, a siderophore first isolated from *Actinosynnema mirum* DSM 43827 (Giessen et al., 2012). The *Streptomyces* A7 genome lacks three genes in the mirubactin BGC – *Amir_2716*, *2715* and *2714*. These encode a LuxR-family regulator, a luciferase-like monooxygenase, and a TetR-family regulator. Region 33a does possess a gene assigned *DKB30_32745* by antiSMASH, predicted to encode a GntR-family regulator in a similar relative location to *Amir_2714* – 4252bp from the phospho-2-dehydro-3-deoxyheptonate aldolase *Amir_2717* in the mirubactin BGC compared to 4549bp in *Streptomyces* A7, suggesting it could fill a similar role. However, other than this similarity, there is little relation between the genes encoded by *Streptomyces* A7 and those in the mirubactin BGC. In place of *Amir_2716* and *2715*, *Streptomyces* A7 processes a gene assigned *DKB30_32730* by antiSMASH, predicted to encode a methionyl-tRNA formyltransferase, another assigned *DKB30_32735*, predicted to amidinotransferase and finally a gene assigned *DKB30_32740*, although it is unknown what this last gene encodes. It may be that Region 33a of *Streptomyces* A7 encodes for a structural analogue of mirubactin. Further study would be required to determine this. Region 33b had 100% similarity to *BGC0001119*, encoding divergolide A/B/C/D in the mangrove tree-associated *Streptomyces sp*. HKI0576. Divergolide A/B/C/D can inhibit *B. subtilis* and *Mycobacterium vaccae* and is toxic to various human cancer cell lines (Ding et al., 2011). Region 37 was predicted to have a 100%

similarity to the *S. coelicolor* A3(2) BGC encoding the siderophore coelichelin (Bentley et al., 2002).

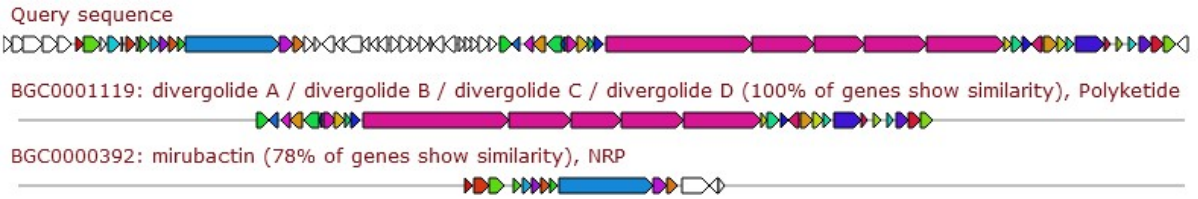


Figure 28 - AntiSMASH Prediction of Region 33. Upon manual review, it was determined that this region contained two smBGCs - one with 100% similarity to the divergolide A/B/C/D encoding BGC from *Streptomyces sp.* HKI0576 and the other 78% similarity to the mirubactin encoding BGC found in S. coelicolor A3(2). These were thus labelled as Regions 33a and 33b, respectively.

Streptomyces A7 was consistently unable to grow on two media – SM6 and SM15, and only sporadically able to grow on a further three – SM19, SM32 and minNAG. When able to grow, *Streptomyces* A7 was able to inhibit *B. subtilis* on 16 media (SFM, SFMNAG, SFMSB, YP, YPD, SPY, MYM, YEME, IMA, GYM, SM5, SM7, SM12, SM14, SM20, GYM + 2ug/mL streptomycin and GYM + 10ug/mL streptomycin), *E. coli* on a single medium (GYM + 2ug/mL streptomycin) and *C. albicans* on three (minNAG, GYM + 2ug/mL streptomycin, GYM + 10ug/mL streptomycin), as presented in

Table 22. Given the presence of Region 33b, which has 100% similarity to a BGC encoding divergolide A/B/C/D, it seems likely that a divergolide-class compound is responsible for the *B. subtilis* activity observed here. It is possible that this also explains the ability for *Streptomyces* A7 to inhibit the growth of *C. albicans* and *E. coli*. However, this activity required the use of streptomycin supplementation as part of a ribosomal engineering technique. Given

that this may induce a point mutation in the *str*, *rpoB* or *rpsL* genes in the ribosome, it may be that the wild-type of this strain cannot inhibit these strains because the antimicrobial is not produced in sufficient concentrations.

Table 22 - Bioassay Results of *Streptomyces* A7 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Agar Name	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	P	P	N	N	N	N
SFMNAG	P	P	N	N	N	N
SFMSB	P	P	N	N	N	N
YP	S	S	N	N	N	N
YPD	S	S	N	N	N	N
SPY	P	P	N	N	N	N
FML	N	N	N	N	N	N
MYM	P	P	N	N	N	N
YEME	S	S	N	N	N	N
IMA	N	N	N	N	N	N
GYM	S	N	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N
SM5	S	P	N	N	N	N
SM6	F	F	F	F	F	F
SM7	P	P	N	N	N	N
SM12	N	S	N	N	N	N
SM14	P	N	N	N	N	N

SM15	F	F	F	F	F	F
SM18	N	N	N	N	N	N
SM19	F	F	F	F	N	N
SM20	P	P	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	N	N	N
SM32	F	F	F	F	N	N
MinNAG	F	F	N	N	S	S
GYM + 10µg/mL streptomycin	S	S	S	S	S	S
GYM + 2µg/mL streptomycin	S	S	N	N	S	S
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.10 AntiSMASH Analysis of *Streptomyces* KY4

Analysis of *Streptomyces* KY4 revealed 28 smBGCs; eight of these were predicted to be PKS encoding smBGCs (Regions 1.4, 1.5, 1.6, 1.8, 1.11, 1.13, 1.23 and 3.1), three RiPP (Regions 1.15, 1.19 and 1.21), three NRPS (Regions 1.2, 1.18 and 1.22), four terpenes (Regions 1.12, 1.16, 1.20 and 1.24), two hybrid PKS/NRPS (Regions 1.1 and 3.2) and eight other (Regions 1.3, 1.7, 1.9, 1.10, 1.14, 1.17, 1.25 and 1.26). 11 smBGCs had high homology to previously reported smBGCs, this is presented in Appendix 7.2.10. Region 1.2 possessed 100% similarity with the smBGC encoding coelibactin in *S. coelicolor* A(3)2, and Region 1.4 for the germicidin encoding BGC present in *S. argillaceus* (Bentley et al., 2002; Becerril et al., 2018). An 86% similarity was predicted between Region 1.10 and the siderophore desferrioxamine smBGC, while Regions 1.12 had 1.16 had 100% similarities to albaflavenone and geosmin, respectively. Region 1.9 had 100% similarity to the *S. coelicolor* A3(2) smBGC encoding the lantipeptide SapB. SapB was first described as a spore-associated peptide required for morphology regulation (Nguyen et al., 2002). It has since additionally been determined to have many similarities to a type A lantibiotic – ribosomally synthesised oligopeptides that possess antimicrobial activity. Type A lantibiotics are synthesised in a 'pre-peptide' form and then extensively modified post-translation, often by dehydrating serine and threonine residues, allowing them to form bridges with cystine residues (Kodani et al., 2004). These can then form pores in the plasma membrane of the cell, allowing for the leakage of ions, salts, amino acids and nucleotides, resulting in the cessation of biosynthetic processes and death of the cell (Sahl and Bierbaum,

2003). Despite these similarities, SapB is unusual among *Streptomyces* lantipeptides in not exhibiting antimicrobial activity in any of a range of Gram-positive and Gram-negative bacteria, including *E. coli* and *B. subtilis* (Kodani et al., 2004). The remaining four regions with 100% homology to previously reported smBGCs – Regions 1.20 (hopene), 1.22 (coelichelin), 1.23 (alkylresorcinol) and 1.24 (isorenieratene) – have all been discussed previously.

When bioassays were performed using *Streptomyces* KY4, it was observed that the ability to inhibit *B. subtilis* was restricted to FML, whilst *C. albicans* inhibition was restricted to SM15 and GYM+2ug/mL streptomycin – this is presented in Table 23. Given the presence of an albaflavenone encoding smBGC in the genome, it is perhaps surprising not to see activity against *B. subtilis* in a broader variety of media. This suggests that this smBGC in *Streptomyces* KY4 is difficult to elicit in lab conditions and that other potential antimicrobial-encoding smBGC are either lacking or are equally challenging to elicit activity from.

Table 23 - Bioassay Results of *Streptomyces* KY4 When Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Agar Name	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	S	S	N	N	N	N
MYM	N	N	N	N	N	N
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N
SM5	N	N	N	N	N	N
SM6	N	N	N	N	N	N
SM7	N	N	N	N	N	N
SM12	N	N	N	N	N	N
SM14	N	N	N	N	N	N
SM15	N	N	N	N	S	S

SM18	N	N	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	N	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	N	N	N
SM32	N	N	N	N	N	N
minNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	S	S
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.11 AntiSMASH Analysis of *Streptomyces* FG1

As presented in Appendix 7.2.11, *Streptomyces* FG1 was determined to encode 35 smBGCs. 10 of these were predicted to encode PKSs (Regions 3, 6, 8, 11, 13, 19, 20, 29, 30 and 34), four RiPPs (Regions 1, 12, 17 and 18), seven NRPSs (Regions 5, 9, 14, 22, 31, 32 and 35), four terpene (Regions 2, 10, 16 and 33), one NRPS/PKS hybrid (Region 7) and nine other (Regions 4, 15, 21, 23, 24, 25, 26, 27 and 28). Region 25 had characteristics of both a RiPP and an NRPS encoding BGC, but, due to difficulties determining which it resembled more closely, it was placed in the 'other' category. 12 smBGCs encoded by *Streptomyces* FG1 were determined to have a high similarity to previously reported BGCs.

Region 2 was predicted to possess a 100% similarity to the isorenieratene BGC from *S. griseus* NBRC 13350, discussed previously. Region 4 had 100% similarity to the melanin encoding smBGC, also first discovered in *S. griseus* NBRC 13350, encoding a pigment that protects the bacterium from ultraviolet radiation, heavy metals and oxidative stress (Ohnishi et al., 2008; Pavan, López and Pettinari, 2020). Region 5 was predicted to have 91% similarity with the valinomycin encoding smBGC in *Streptomyces sp.* CBMAI 2042 is an ionophore highly selective for potassium ions (Rose and Henkens, 1974; Paulo et al., 2019). In this way valinomycin can act both as a potent antibacterial agent and as a broad-spectrum antiviral, with potential uses including the treatment of severe acute respiratory syndrome coronavirus-2 (D. Zhang et al., 2020). *Streptomyces* FG1 does lack *stan6724*, a gene encoding a protein with unknown function. This makes it difficult to determine the likelihood that valinomycin is being

produced; however, given the relatively small size of the gene, being 452bp, and the high homology of the rest of the region with the BGC, it seems likely that a compound very similar in structure to valinomycin is encoded, if not valinomycin itself. A third region, Region 7, has a 100% similarity to an smBGC first reported in *S. griseus* NBRC 13350, encoding a polycyclic tetramate macrolactam (PTM) designated SGR after the organism it was initially isolated from. Although some PTMs possess antimicrobial activity, it is unknown if SGR-PTM does. PTMs are found in many *Streptomyces* species, and the smBGCs encoding them can be traced back to a single origin, frontalamide, with which the region has an 85% homology (Blodgett et al., 2010; Luo et al., 2013).

Region 8 had 100% similarity for the bafilomycin B1 smBGC from *S. Iohii*, a plecomacrolide that is used as an anti-osteoporotic and can be used in combination with other drugs to treat *Cryptococcus neoformans*, an opportunistic fungal pathogen of the central nervous system (Del Poeta et al., 2000; Zhang et al., 2013). Despite this, clinical use of bafilomycin B1 remains rare due to its low therapeutic index. Another lanthipeptide was predicted to be encoded by Region 17, which had a 100% similarity to the AmfS-encoding smBGC previously discussed. Region 18 had a 100% similarity to the keywimysin-encoding BGC in *Streptomyces sp*. NRRL F-5702. Keywimysin is a lasso-peptide, a class of RiPPs which can possess a wide range of biological activities, including antimicrobial in some cases, which can be highly variable in structure. Antimicrobial lassopeptides include capistruin, a Gram-negative RNA polymerase inhibitor first isolated from *Burkholderia thailandensis* E264 and lassomycin, isolated from the actinobacteria *Lentzea kentuckyensis sp.* that targets ClpC1 ATPase in *Mycobacterium tuberculosis* (Knappe et al., 2008; Gavrish et al., 2014; Hegemann et al., 2015). Keywimsyin has not been isolated; the smBGC was reported by Rapid ORF Description and Evaluation Online, a bioinformatic tool used to predict the smBGC encoding and structures of RiPPs (Tietz et al., 2017). As this is a prediction, not a confirmed smBGC, it would be interesting to attempt to isolate the compound encoded by Region 18 to determine how it compares to the predicted smBGC. Region 28 was predicted to have 100% homology with ectoine. Region 31 had an 81% similarity to the coelichelin encoding BGC in *S. coelicolor* A3(2) (Bentley et al., 2002). Region 31 lacked *sco0499*; a gene predicted to encode a formyl transferase. Given this, it seems likely that Region 31 encodes a structural analogue of coelichelin, although further study would be needed to confirm this. Region 32 was predicted to have a 94% similarity to

the BGC in *Streptomyces sp.* ATCC 700974 encodes the siderophore streptobactin (Patzer and Braun, 2010; Matsuo et al., 2011). Finally, Region 33 had 100% similarity to the geosmin BGC.

When challenged by *B. subtilis*, *Streptomyces* FG1 could inhibit it on 13 media (YP, YPD, SPY, FML, MYM YEME GYM, SM12, SM14, SM19, SM20, SM25, and GYM+10µg/mL streptomycin). However, activity against *E. coli* and *C. albicans* were more limited – inhibition only being observed on three (FML, YEME and GYM+10µg/mL streptomycin) and six (SFM, FML, YEME, SM20, GYM+10µg/mL streptomycin and GYM+2µg/mL streptomycin) media respectively. This is presented in Figure 29 and

Table 24. *Streptomyces* FG1 failed to grow on SM15. It is possible that the product of Region 5, which is predicted to be similar to valinomycin, could be responsible for this activity since *E. coli* activity is only occasionally seen on the same media as *B. subtilis* activity, which can be explained if the concentrations only occasionally were high enough to inhibit *E. coli*. If a higher concentration is needed to inhibit *E. coli* than *B. subtilis*, it may be that the smBGC in Region 5 is only upregulated sufficiently on a sub-set of the media used. Alternatively, Region 18, predicted to encode keywimysin, may be responsible for some or all of the observed antimicrobial activity. As the bioactivity of keywimysin itself has not been determined, it is possible that it does not possess antimicrobial activity. If keywimysin is antimicrobial, it may, in combination with the product encoded by Region 5, explain the antimicrobial profile of *Streptomyces* FG1, where *B. subtilis* activity is seen in a broader variety of media than *E. coli* or *C. albicans* activity. The fact that *E. coli* inhibition was observed only on some of the media *B. subtilis* activity could be explained if either the product encoded by Region 5 or keywimysin only inhibited *B. subtilis* whilst the other that was capable of inhibiting *E. coli* was elicited on only a subset of the media that the *B. subtilis* inhibitor was elicited upon.

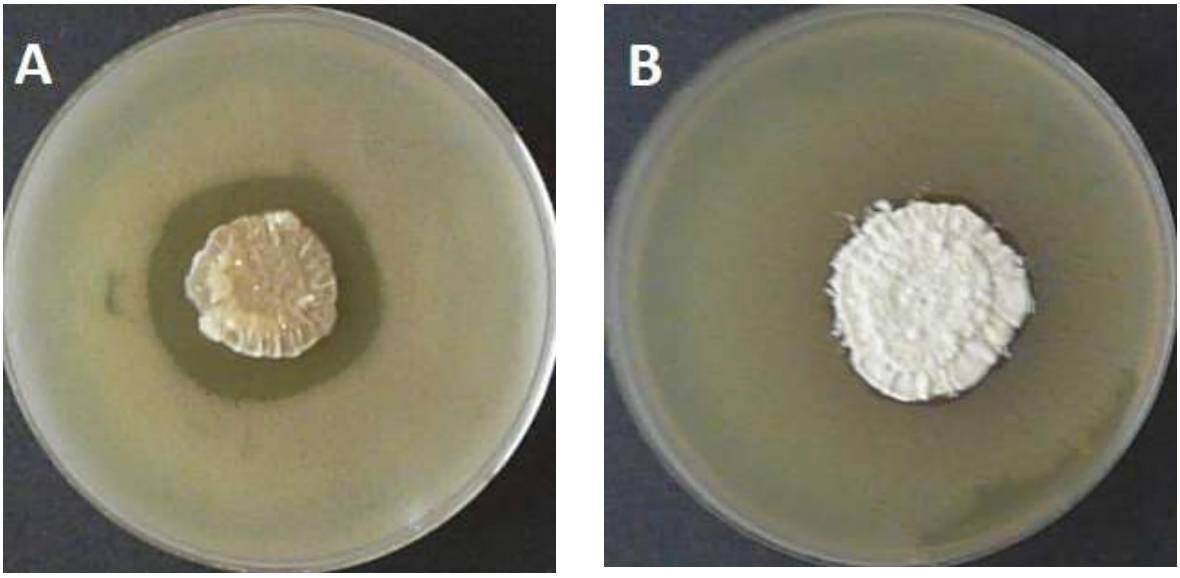


Figure 29 – Bioassays of *Streptomyces* FG1 When Challenged by A - *B. subtilis* on YP, B – *C. albicans* on SM20. These were both assigned 'S'.

Table 24 - Bioassay Results of *Streptomyces* FG1 When Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Agar Name	Target Strain					
	B. subtilis		*E. coli*		*C. Albicans*	
SFM	N	N	N	N	S	S
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	S	S	N	N	N	N
YPD	S	S	N	N	N	N
SPY	S	S	N	N	N	N
FML	P	P	S	S	S	S
MYM	S	S	N	N	N	N
YEME	S	S	P	P	N	S
IMA	N	N	N	N	N	N
GYM	S	S	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N

SM5	N	N	N	N	N	N
SM6	N	N	N	N	N	N
SM7	N	N	N	N	N	N
SM12	N	S	N	N	N	N
SM14	S	S	N	N	N	N
SM15	F	F	F	F	F	F
SM18	N	N	N	N	N	N
SM19	S	S	N	N	N	N
SM20	S	S	N	N	S	S
SM25	N	S	N	N	N	N
SM30	N	N	N	N	N	N
SM32	N	N	N	N	N	N
minNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	S	S	S	S	S	S
GYM + 2µg/mL streptomycin	N	N	N	N	S	N
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.12 AntiSMASH Analysis of *Streptomyces* KY1

Streptomyces KY1 was predicted to have 19 smBGCs. As presented in Appendix 7.2.12, five of these were predicted to encode PKS BGCs (Regions 1.1, 1.6, 1.8, 1.9 and 3.2), one RiPP (Region 1.7), two NRPS (Regions 1.3 and 1.11), three terpenes (Regions 1.2, 1.10 and 1.14), three hybrid PKS/NRPS (Regions 1.15, 3.1 and 5.1) and five classified as others (Regions 1.4, 1.5, 1.12, 1.13 and 2.1). Seven of these were determined to have high homology to previously reported BGCs. *Streptomyces* KY1 Region 1.2 and B2 Region 1.19 were both predicted to have an 85% similarity to the smBGC from *S. griseus* NBRC 13350 encoding isorenieratene, and both lacked *sgr6825*, encoding a methyltransferase. Despite this, the two regions are different to each other. In Region 1.19 of *Streptomyces* B2, there is a 23bp gap between *sgr6826* and *sgr6827*, whereas, in *Streptomyces* KY1, this gap is 1401bp long and was predicted to contain three genes - a nuclear transport factor 2 family protein first discovered in *Streptomyces sp*. CS227 that facilitates protein transport into the nucleus, a transactional regulator from the same *Streptomyces sp*. CS227 strain and a hypothetical protein that is either a multispecies protein found in many *Streptomyces* species or, more specifically, hypothetical protein BU198_18435 from *Streptomyces sp*. CBMA156. It appears unlikely that

Streptomyces KY1 and B2 encode the same secondary metabolite or that Region 1.2 of *Streptomyces* KY1 encodes for isorenieratene, although it is possible that this region still encodes a terpene-class compound.

Region 1.4 was predicted to have a 100% similarity to the ectoine-encoding smBGC, 1.5 to the desferrioxamine B smBGC, 1.10 to the geosmin smBGC and 1.15 to the SGR-PTM smBGC, all of which have been discussed previously. Region 3.1 was predicted to have 100% similarity to the smBGC encoding antimycin. This antimycin smBGC has been observed in several actinomycetes, including the *Acromyrmex octospinosus* symbiont *Streptomyces* S4 (Seipke et al., 2011). Antimycin is toxic to many eukaryotic cells, is used as a piscicide in fisheries, and has antifungal properties. This lethality is caused by antimycin interacting with the mitochondrial membrane protein Bcl-xL, preventing it from dimerising with Bcl-2 leading to the inhibition of electron transport across the mitochondrial membrane, leading to apoptosis of the cell (Tzung et al., 2001; Seipke et al., 2011). Region 3.2 was predicted to have a 90% similarity to the candicidin-encoding smBGC in *Streptomyces sp*. FR-008 but lacked the GDP-mannose-4, 6-dehydratase encoding *fscMIII* gene, responsible for the biosynthesis of mycosamine – a sugar moiety attached to an intermediate in the candicidin pathway. This suggests a structural analogue is encoded by *Streptomyces* KY1, which possesses an alternative sugar moiety (Chen et al., 2003).

As presented in

Table 25, *Streptomyces* KY1 was observed inhibiting *B. subtilis* on five media (FML, IMA, GYM, SM18, SM25) and *C. albicans* on five media (MYM, GYM, SM6, SM25, GYM+10µg/mL Streptomycin). *E. coli* inhibition was not observed on any media, and *Streptomyces* KY1 could not grow on SM15. Region 1.15, predicted to encode SGR-PTM, may explain the antimicrobial activity observed – although, as previously discussed, it is unknown if SGR-PTM possesses antimicrobial properties. The inhibition of *C. albicans* is probably related to Region 3.2, likely encoding a candicidin analogue, and this BGC is rarely elicited. It is also possible that Region 1.5, encoding desferrioxamine B, is sequestering iron and preventing the growth of *C. albicans*.

Table 25 - Bioassay Results of *Streptomyces* KY1 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Agar Name	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	S	S	N	N	N	N
MYM	N	N	N	N	P	P
YEME	N	N	N	N	N	N
IMA	S	S	N	N	N	N
GYM	S	S	N	N	P	P
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N
SM5	N	N	N	N	N	N
SM6	N	N	N	N	S	S
SM7	N	N	N	N	N	N
SM12	N	N	N	N	N	N
SM14	N	N	N	N	N	N
SM15	F	F	F	F	F	F
SM18	S	S	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	N	N	N	N	N
SM25	S	S	N	N	S	S
SM30	N	N	N	N	N	F
SM32	N	N	N	N	N	N
minNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	S	S
GYM + 2µg/mL streptomycin	N	N	N	N	N	N
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.13 AntiSMASH Analysis of *Amycolatopsis* UM15

Analysis of *Amycolatopsis* UM15 revealed 33 smBGCs, six encoding PKS (Regions 1.6, 1.7, 1.10, 1.12, 1.27 and 3.2), a single RiPP (Region 3.3), four NRPS (Regions 1.1, 1.13, 1.18 and 2.1), five terpenes (Regions 1.2, 1.9, 1.23, 3.1 and 3.4), five hybrid PKS/NRPS (Regions 1.5, 1.11, 1.16, 1.22 and 4.1), with 12 other (Regions 1.3, 1.4, 1.8, 1.14, 1.15, 1.17, 1.19, 1.20, 1.21, 1.25, 1.25 and 1.26). This is presented in Appendix 7.2.13. Of these, four had high homology to previously discovered smBGCs. This is fewer than expected given the high number of smBGC encoded in the genome, suggesting that *Amycolatopsis* biosynthetic pathways are understudied compared to those from *Streptomyces* species.

Region 1.10 had a 96% homology to the macrotermycin encoding BGC first isolated from the *Macrotermes natalensis*-associated *Amycolatopsis sp.* M39. *M. natalensis* is a fungus-growing termite native to South Africa that cultivates fungi from the *Termitomyces* genus, similarly to the leafcutter ants studied here. The *Termitomyces* fungal garden is similarly vulnerable to invasion by *Pseudoxylaria*, similarly to how *Leucocoprineae* is invaded by *Escovopsis* species and, similarly to the ant species studied here, termites co-opt antimicrobial-producing actinomycetes. Macrotermycin has been shown to be effective at inhibiting *B. subtilis, Staphylococcus aureus* and *C. albicans* (Visser et al., 2011; Beemelmanns et al., 2017). *Amycolatopsis* UM15 lacked a dehydrogenase involved in sugar biosynthesis as part of the larger macrotermycin biosynthetic pathway. This may mean a different sugar is used in the biosynthetic pathway of Region 1.10, and a structural analogue is produced. Regions 1.23 and 1.25 were predicted to have a 100% similarity to the previously discussed geosmin and ectoine smBGCs, respectively. Region 3.4 has a 100% similarity for the smBGC encoding 2-methylisobornel from the cyanobacteria *Pseudanabaena sp*. dqh15. Curiously, whilst 2-methylisobornel has been sequenced in *Saccharopolyspora erythraea* NRRL23338, there was no significant similarity between the *S. erythraea* smBGC and Region 3.4 in *Amycolatopsis* UM15 when analysed using NCBI BLAST (Oliynyk et al., 2007; Wang et al., 2011). This extends to other 2-methylisobornel smBGCs sequenced from *Streptomyces* species (Komatsu et al., 2008). 2-methylisobornel is responsible for many water fouling events, affecting the taste and smell of drinking water (Jüttner and Watson, 2007). Despite its importance as a water and fish fouling agent, the biological function of 2-methylisobornel is unknown.

As presented in

Table 26, *Amycolatopsis* UM15 showed activity against *B. subtilis, E. coli* and *C. albicans* on a wide variety of media –on 18 (SFM, SFMNAG, SFMSB, FML, YEME, GYM, Minimal, SM3, SM5, SM6, SM7, SM12, SM14, SM20, SM25, SM30, SM32 and MinNAG), nine (SFM, SFMNAG, SFMSB, FML, YEME, SM3, SM5, SM12, SM30 and MinNAG) and 10 (SFM, SFMNAG, SFMSB, FML, YEME, IMA, GYM, SM5, MinNAG and GYM + 2μg/mL streptomycin) media, respectively. Additionally, *Amycolatopsis* UM15 only grew intermittently on SM15 and SM18. This antimicrobial activity may be related to Region 1.10, although macrotermycin has not been tested against Gram-positive pathogens, so it is unclear if this fully explains the activity observed. In addition to the usual bioassays performed on all strains, additional bioassays were performed on *Amycolatopsis* UM15 against MRSA on SFM, SFMNAG, FML and GYM – all media upon which *Amycolatopsis* UM15 demonstrated the ability to *inhibit B. subtilis*. In none of these conditions was inhibition of MRSA observed. This may mean that Region 1.10 is not responsible for the antimicrobial activity observed, as macrotermycin was shown to inhibit MRSA strains, or it may be the potential structural change caused by the lack of dehydrogenase enzyme in *Amycolatopsis* UM15 led to reduced ability to inhibit MRSA growth.

Table 26 - Bioassay Results of *Amycolatopsis* UM15 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Agar Name	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	P	P	P	P	S	N
SFMNAG	P	P	S	P	P	P
SFMSB	P	P	P	P	P	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	P	P	S	S	S	P
MYM	N	N	N	N	N	N
YEME	P	P	S	S	S	S
IMA	N	N	N	N	S	S
GYM	P	P	N	N	P	S
Minimal	N	P	N	N	N	N
SM3	S	S	S	S	N	N
SM5	S	N	N	N	S	N
SM6	S	S	N	N	N	N
SM7	S	S	N	N	N	N
SM12	S	S	S	S	N	N
SM14	P	P	N	N	N	N
SM15	N	N	F	F	N	F

SM18	N	F	N	F	N	F
SM19	N	N	N	N	N	N
SM20	N	S	N	N	N	N
SM25	P	P	N	N	N	N
SM30	P	P	S	F	N	N
SM32	S	S	N	N	N	N
MinNAG	P	P	S	S	S	S
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	S	S
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.14 AntiSMASH Analysis of *Amycolatopsis* FG22

Amycolatopsis FG22 was predicted to possess the highest number of smBGCs of any of the rare actinomycetes studied here with 35, presented in Appendix 7.2.14. Of these, 11 were predicted to encode PKS (Regions 5, 8, 9, 14, 15, 16, 18, 22, 26, 34 and 35), one RiPP (Region 13), six NRPS (Regions 3, 21, 23, 27, 28 and 29), three terpene (Regions 11, 19 and 31), one hybrid NRPS/PKS (Region 7) and 13 other (Regions 1, 2, 4, 6, 10, 12, 17, 20, 24, 25, 30, 32 and 33). Six regions had high homology to previously reported smBGCs. This, again, is lower than expected for a *Streptomyces* genome containing this many smBGCs. Region 10 was predicted to have 100% homology for an ectoine-encoding smBGC, and Region 11 for geosmin. Region 13 had a 100% similarity to the *Saccharopolyspora erthraea* NRRL 2338 smBGC encoding erythreapeptin 3, 4, 5, 6, 7, 8 and 9. Erythreapeptin is described as a type III lantibiotic in the literature; however, there is no reference to any antimicrobial properties. Therefore, it may be more appropriate to describe erythreapeptin as a lantipeptide, similar to SapB from *S. coelicolor* and SapT from *S. tendae*, which have a primarily morphogenetic role as opposed to the primarily antimicrobial role of type A and B lantibiotics, despite both lantipeptides and lantibiotics processing lanthionine residues (Kodani et al., 2005; Dischinger et al., 2009; Völler et al., 2012; Kashyap, 2019)

Region 17 had high homology to nine different glycopeptide-encoding smBGC, all of which have previously been discovered in *Amycolatopsis* species; 88% similarity to the keratinimicin A/B/C/D smBGC from *A. keratiniphila* (Xu et al., 2019), 95% similarity to the nogabecin from

A. keratiniphila subsp. *Nogabecina* (Wink et al., 2003), 86% similarity to the decaplanin BGC from *A. regifaucium* (Tan et al., 2007), 89% similarity to the avoparcin BGC from *A. coloradensis* (Labeda, 1995), 91% similarity to the decaplanin BGC from *A. decaplanina* DSM 44594 (Wink et al., 2004), 92% similarity to the ristomycin BGC from *A. japonica* MG417-CF17 (Spohn et al., 2014), 81% similarity to the balhimycin BGC from *A. balhimycina* DSM 5908 and, 88% similarity to the vancomycin BGC from *A. orientalis* (Xu et al., 2014). This strongly implies that Region 17 encodes a glycopeptide-class compound, likely to be an antimicrobial agent against Gram-positive bacteria. Additionally, although Gram-negative bacteria are typically intrinsically resistant to glycopeptide antibiotics, as they are unable to cross the outer membrane, they can be effective in inhibiting the growth of Gram-negative bacteria when combined with other antimicrobials or if groups that resemble detergent moieties are attached to the molecule. Although these moieties are generally attached synthetically, it may be possible for versions of compounds containing similar properties to be produced biosynthetically (Acharya et al., 2022). Region 28 was predicted to have 100% homology to the smBGC encoding albachelin, a siderophore, from *A. alba* DSM 44262 (Kodani et al., 2015). Finally, Region 22 was predicted to have 100% similarity to the smBGC encoding 2-methylisoborneol, previously discussed.

As

Table 27 shows, *Amycolatopsis* FG22 demonstrated the ability to inhibit *B. subtilis* on 17 media (SFM, SFMNAG, SFMSB, FML, YEME, GYM, SM3, SM5, SM7, SM15, SM25, SM30, SM32, GYM+10µg/mL streptomycin, ISP4 and SFMCasAA), *E. coli* on 10 (SFMNAG, FML, MYM, IMA, SM7, SM15, SM25, SM32, GYM+10µg/mL streptomycin and SFMCasAA) and *C. albicans* on 12 (SFM, SFMNAG, SFMSB, SPY, FML, MYM, YEME, GYM, SM7, SM15, SM32 and SFMCasAA). However, as shown in Figure 30, when SFM was supplemented by 50 or 500µM $FeCl_3$, activity against *C. albicans* was lost. This implies that this activity is caused by a siderophore sequestering iron rather than an antifungal compound. Further work is needed to confirm

this, as iron supplementation also caused the loss of black discolouration of the media, which could either be a black-colour siderophore or an unrelated pigment. Interestingly, *Amycolatopsis* FG22 consistently failed to grow on GYM supplemented with 2µg/mL streptomycin but grew successfully and showed antimicrobial activity against *B. subtilis* and *E. coli* when cultivated on GYM supplemented with 10µg/mL streptomycin. One possible explanation is that the BGC that conveys resistance to streptomycin is not sufficiently upregulated at lower concentrations of streptomycin, but increasing the concentration increases the expression of the resistance genes. If the BGC conveying resistance only does so incidentally, for example, by encoding an efflux pump for the product of the BGC that also can efflux streptomycin, then this may explain why it is not upregulated at low levels of streptomycin.

Table 27 - Bioassay Results of *Amycolatopsis* FG22 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium.

Agar Name	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	P	P	N	N	S	S
SFMNAG	P	P	S	S	S	S
SFMSB	P	P	N	N	P	P
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	S	S
FML	P	P	S	S	S	P
MYM	N	N	P	S	P	P
YEME	P	P	N	N	S	S
IMA	P	P	S	S	N	N
GYM	P	P	N	N	S	S
Minimal	N	N	N	N	N	N
SM3	P	P	N	N	N	N
SM5	S	N	N	N	N	N

SM6	N	N	N	N	N	N
SM7	P	P	P	P	P	P
SM12	N	N	N	F	N	N
SM14	N	N	N	N	N	N
SM15	P	P	P	P	P	P
SM18	N	N	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	N	N	N	N	N
SM25	S	S	S	N	N	N
SM30	S	N	N	N	N	N
SM32	P	P	P	P	N	P
MinNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	S	S	P	S	N	N
GYM + 2µg/mL streptomycin	N	F	F	F	F	F
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	S	S	N	N	N	N
SFM+CasAA	S	S	S	S	S	S

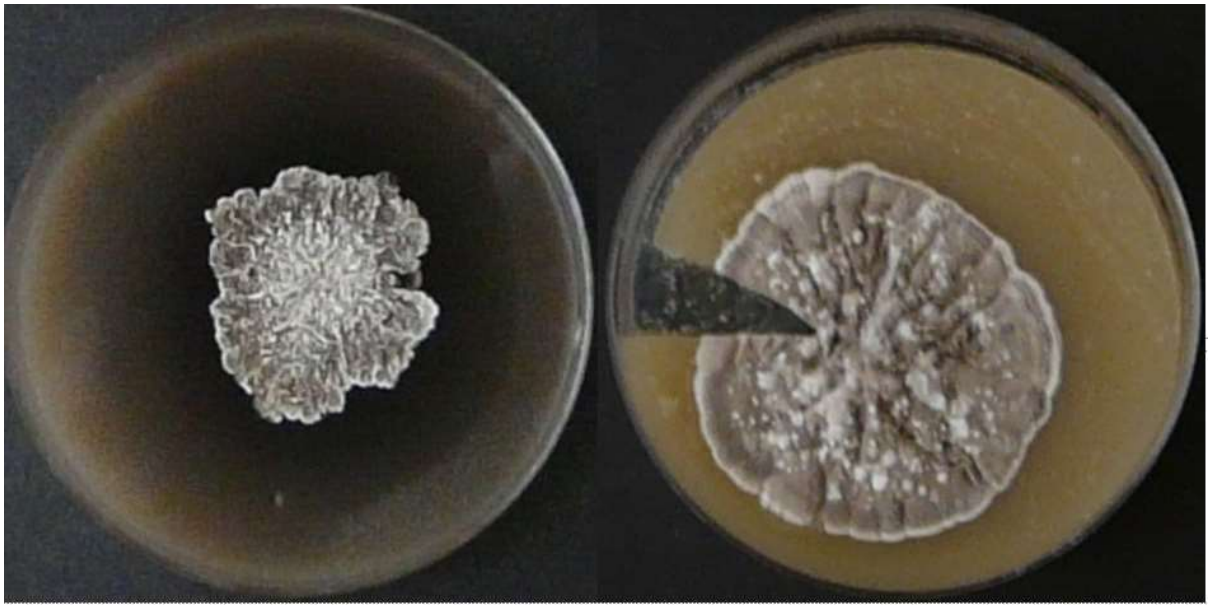


Figure 30 - Bioassays of *Amycolatopsis* FG22 when challenged by *C. albicans* on SFM (left) or SFM supplemented with 50µM $FeCl_3$ (Right). Iron supplementation reduced the inhibition of *C. albicans* and the loss of black media discolouration.

3.3.15 AntiSMASH Analysis of *Agrococcus* A6

Agrococcus A6 had the smallest genome of the strains studied here, with 3.12Mbpm and the least smBGC, with only three being predicted. As presented in Appendix 7.2.15, Regions 1 and 3 were predicted to encode PKSs and Region 2 was assigned to the 'other' category. None of

these regions had significant similarity to previously discovered smBGCs. It is unclear if *Agrococcus* A6 does indeed encode so few smBGCs or if antiSMASH is limited in its ability to predict BGCs from the genus, given that the NCBI database only contains 32 *Agrococcus* genomes (NCBI, 2022). The relatively small genome size of *Agrococcus* A6 means both possibilities are viable. Despite this lack of smBGCs, *Agrococcus* A6 inhibited *B. subtilis* on three media (GYM, SM6 and SM25), *E. coli* on GYM + 10µg/mL streptomycin and *C. albicans* on GYM + 2µg/mL streptomycin. Table 28 presents a summary of this activity. *Agrococcus* A6 could not grow on the broadest variety of media used, including YP, YPD, SM19, SM32 and MinNAG, while only sporadically growing on minimal, SM3, SM6, SM7, SM18 and SM30. These media were optimised for eliciting *Streptomyces* cryptic smBGCs by applying stress factors rather than maximising growth or sporulation. It is possible that the stressful conditions caused by some of these media were too much for *Agrococcus* A6, which was not able to survive. This suggests that *Agrococcus* A6 is the strain with the smallest ecological niche of the strains studied here.

Table 28 - Bioassay results of *Agrococcus* A6 when challenged by select pathogens on various media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Agar Name	Target Strain					
	B. subtills		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	F	F	F	F	F	F
YPD	F	F	F	F	F	F
SPY	N	N	N	N	N	N
FML	F	F	F	N	F	F
MYM	N	N	N	N	N	N
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	S	S	N	N	N	N
Minimal	F	F	N	N	F	N
SM3	N	F	N	N	N	N
SM5	N	N	N	N	N	N
SM6	S	N	N	N	F	N
SM7	N	N	N	N	F	N
SM12	N	N	N	N	N	N
SM14	N	N	N	N	N	N

SM15	N	N	N	N	N	N
SM18	F	F	N	F	N	N
SM19	F	F	F	F	F	F
SM20	N	N	N	N	N	N
SM25	N	S	N	N	N	N
SM30	F	F	N	N	N	N
SM32	F	F	F	N	F	F
MinNAG	F	F	F	F	F	F
GYM + 10µg/mL streptomycin	N	N	P	P	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	P	P
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.16 AntiSMASH Analysis of *Tsukamurella* FG11

The antiSMASH analysis of *Tsukamurella* FG11, displayed in Appendix 7.2.16, predicted 14 smBGCs, none of which had high homology to previously reported BGCs. One of these (Region 14) was predicted to encode a PKS, three NRPS (Regions 1, 2 and 13), three terpenes (Regions 3, 9 and 11), and one hybrid NRPS/PKS (Region 7) with the remaining six being other (Regions 5, 7, 8, 10 and 12). Region 6 likely encodes ectoine, despite antiSMASH not reporting a higher than 75% homology for any previously reported smBGC. Region 6 has high homology for the hydroectoine encoding smBGC from *S. chrysomallus* but lacks ThpD, which converts ectoine into hydroecotoine. Given this lack of ThpD, the biosynthetic pathway would end at ectoine (Prabhu et al., 2004).

Tsukamurella FG11 was not observed to inhibit *E. coli* on any medium but could inhibit *B. subtilis* on MYM, SM12, SM20 and SM32, although not consistently. *C. albicans* was inhibited on YP, YPD and MYM, as summarised in

Table 29. This bioactivity cannot be clearly linked to any predicted *Tsukamurella* FG11 smBGCs.

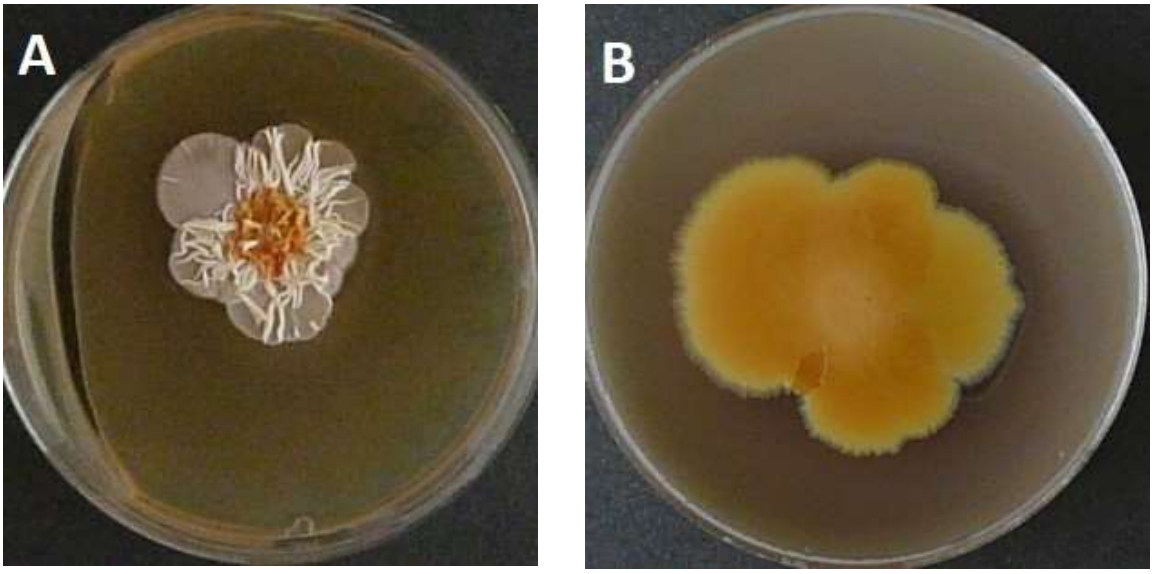


Figure 31 – Bioassays of *Tsukamurella* FG11 when Challenged by *B. subtilis* on A –SM32, B – MYM. These were assigned 'N' and 'S' respectively. It appears that in image B, sporulation had not occurred, suggesting this antimicrobial is produced earlier in the lifecycle,

Table 29 - Bioassay Results of *Tsukamurella* FG11 when Challenged by Select Pathogens on Various Media A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Agar Name	Target Strain					
	B. subtills		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	F	F	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	S	S
YPD	N	N	N	N	S	S
SPY	N	N	N	N	N	N
FML	N	N	N	N	N	N
MYM	S	S	N	N	S	S

YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	N	N
SM5	N	N	N	N	N	N
SM6	N	N	N	N	N	N
SM7	N	N	N	N	N	N
SM12	S	N	N	N	N	N
SM14	N	N	N	N	N	N
SM15	N	N	N	N	N	N
SM18	N	N	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	S	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	N	N	N
SM32	P	N	N	N	N	N
MinNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	N	N
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

3.3.17 AntiSMASH Analysis of *Tsukamurella* E7

The analysis of *Tsukamurella* E7 revealed 18 smBGCs, none of which were predicted to have high homology to previously discovered clusters. Of the 18 predicted BGCs, three were predicted to encode PKS (Regions 1.2, 1.9 and 2.2), three RiPP (Regions 1.3, 1.5 and 2.3), three NRPS (Regions 1.8, 1.10 and 1.12), two terpenes (Regions 1.7 and 2.1) and seven others (Regions 1.1, 1.4, 1.6. 1.11. 1.13, 1.14 and 2.4). None were predicted to be encoding hybrid PKS/NRPS clusters. Appendix 7.2.17 summarises this information.

Table 30 presents a summary of the bioactivity of *Tsukamurella* E7. *B. subtilis* was inhibited on SM3, SM5, SM32 and SFMCasAA and *C. albicans* on MYM and SM5. *E. coli* was not inhibited on any growth medium. The lack of information on the BGCs encoded by *Tsukamurella* E7 makes it challenging to assign which smBGCs may be responsible for the observed activity.

Table 30 - Bioassay Results of *Tsukamurella* E7 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Agar Name	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	N	F	N	F	N	N
MYM	N	N	N	N	S	S
YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	N	N
Minimal	N	N	N	N	N	N
SM3	S	N	N	N	N	N
SM5	S	N	N	N	S	S
SM6	N	N	N	N	N	N
SM7	N	N	N	N	N	N
SM12	N	N	N	N	N	N
SM14	N	N	N	N	N	N
SM15	N	N	N	N	N	N
SM18	N	N	N	N	N	N
SM19	N	N	N	N	N	N
SM20	N	N	N	N	N	N
SM25	N	N	N	N	N	N
SM30	N	N	N	N	N	N
SM32	S	S	N	N	N	N
MinNAG	N	N	N	N	N	N
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	N	N	N	N	N	N
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N

SFM+CasAA	S	S	N	N	N	N

3.3.18 AntiSMASH Analysis of *Jiangella* S1

The antiSMASH analysis of *Jiangella* S1 predicted 12 smBGCs, as presented in Appendix 7.2.18. Three of these were predicted to encode PKS (Regions 2.1, 2.3 and 2.5), two RiPP (Regions 1.5 and 4.2), three NRPS (Regions 4.4, 4.5 and 4.6) and the remaining five assigned as other (Regions 2.2, 2.4, 2.6, 4.1 and 4.3). Only one of these, Region 2.5, had any similarity to any previously reported BGC with a 100% similarity to alkyl resorcinol from *S. griseus* NBRC 13350, which has been discussed previously.

Table 31 summarises the bioactivity of *Jiangella* S1, including its inability to consistently grow on FML, SM3, SM5, SM7, SM15, SM19, SM25, SM32 and MinNAG. Interestingly, *Jiangella* S1 was still able to grow on many of these media sporadically. Despite this, as seen in Figure 32,

Jiangella S1 could inhibit *B. subtilis* on SM20, SM25 and GYM+2µg/mL streptomycin, *E. coli* on SM25 and *C. albicans* on GYM and GYM+2µg/mL streptomycin.

Table 31 - Bioassay Results of *Jiangella* S1 when Challenged by Select Pathogens on Various Media. A green 'P' designates a zone of inhibition of 10mm or larger was observed, a yellow 'S' a zone of inhibition below 10mm was observed, a red 'N' no zone of inhibition was observed and an orange 'F' if the actinomycete failed to grow on the medium. Both replicates for each indicator strain are presented here in separate columns.

Agar Name	Target Strain					
	B. subtilis		*E. coli*		*C. albicans*	
SFM	N	N	N	N	N	N
SFMNAG	N	N	N	N	N	N
SFMSB	N	N	N	N	N	N
YP	N	N	N	N	N	N
YPD	N	N	N	N	N	N
SPY	N	N	N	N	N	N
FML	F	F	F	N	F	F
MYM	N	N	N	N	N	N

YEME	N	N	N	N	N	N
IMA	N	N	N	N	N	N
GYM	N	N	N	N	P	S
Minimal	N	N	N	N	N	N
SM3	N	N	N	N	F	F
SM5	F	F	F	F	F	N
SM6	N	N	N	N	N	N
SM7	N	N	N	F	F	F
SM12	N	N	N	N	N	N
SM14	N	N	N	N	N	N
SM15	F	F	F	F	F	F
SM18	N	N	N	N	N	N
SM19	F	F	N	N	F	F
SM20	N	P	N	N	N	N
SM25	S	S	S	F	N	N
SM30	N	N	N	N	N	N
SM32	F	F	N	N	F	F
MinNAG	F	F	N	F	F	F
GYM + 10µg/mL streptomycin	N	N	N	N	N	N
GYM + 2µg/mL streptomycin	S	N	N	N	S	S
YP/C	N	N	N	N	N	N
ISP2	N	N	N	N	N	N
ISP4	N	N	N	N	N	N
SFM+CasAA	N	N	N	N	N	N

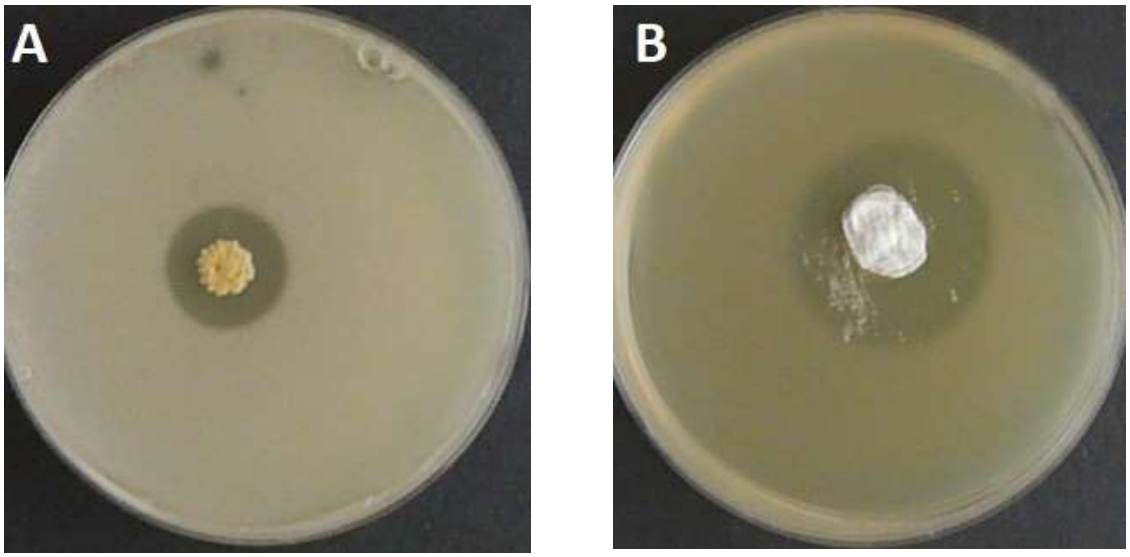


Figure 32 – Bioassays of *Jiangella* S1 on A – GYM when challenged by *C. albicans* B – SM20 when challenged by *B. subtilis.* These were assigned 'S' and 'P' respectively.

3.4 Phylogenetic Analysis of Cultivatable Strains

The 18 strains that could be cultivated had their relationship analysed to determine how related they are to each other. A phylogenetic tree was created with the 16S rRNA gene sequences as per Section 2.4 with all 18 strains included and *S. coelicolor* A3(2) as a model *Streptomyces* organism. 16S rRNA gene sequences were extracted from the whole genome sequences using RNAammer 1.2, with multiple strains possessing multiple 16S rRNA gene sequences, as presented in

Table 32 (Lagesen et al., 2007).

Table 32 - Number of 16S rRNA gene sequences identified from the whole genome sequences. Some of these 16S rRNA gene sequences were duplicates, so the number of unique sequences is also reported.

Strain Designation	Total Number of 16S rRNA Gene Sequences	Number of Unique rRNA Gene Sequences
Pseudonocardia UM4	4	1
Pseudonocardia UM14	3	3
Pseudonocardia UM9	6	2
Pseudonocardia P1	4	3
Streptomyces KY2	6	4
Streptomyces FG4	6	2
Streptomyces B2	6	3
Streptomyces FG7	6	2
Streptomyces A7	6	3
Streptomyces KY4	6	2
Streptomyces FG1	6	4
Streptomyces KY1	7	3
Amycolatopsis UM15	4	2
Amycolatopsis FG22	4	2
Agrococcus A6	1	1
Tsukamurella FG11	2	1
Tsukamurella E7	2	2
Jiangella S1	2	1

B. subtilis ATCC 23857, derived from *B. subtilis* 168, was selected as the outgroup. *B. subtilis* 168 is a commonly used lab strain of *B. subtilis*, and other *B. subtilis* species have previously been used as outgroups for analysis of mixed genera of actinomycetes and analysis of *Streptomyces* species alone (Jose and Jebakumar, 2012; Apsari, Budiarti and Wahyudi, 2019). The General Time Reversible (GTR) model of analysis was deemed to be an acceptable phylogenetic model by FindModel to build a maximum-likelihood (ML) tree from the genome sequences when paired with a gamma rate distribution (Los Alamos National Laboratory, 2016). The GTR model assumes that the mutation rate between bases can vary and that bases can revert to their original state. Whilst a gamma rate distribution allows for different bases to mutate at different rates across the sequenced site (Tavarc, 1986; Groussin et al., 2016). Although there is concern among some that the GTR has a flaw in the underlying mathematics, it remains the most commonly used model for phylogenetic analysis (Sumner et al., 2012). Figure 33 shows the output of this initial phylogenetic analysis, visualised using Interactive Tree of Life v5, including all of the unique 16S rRNA gene sequences from those

strains that processed multiple (Letunic and Bork, 2021). This identified which of the unique 16S rRNA gene sequences would be used in the final analysis so that each strain would only be represented once.

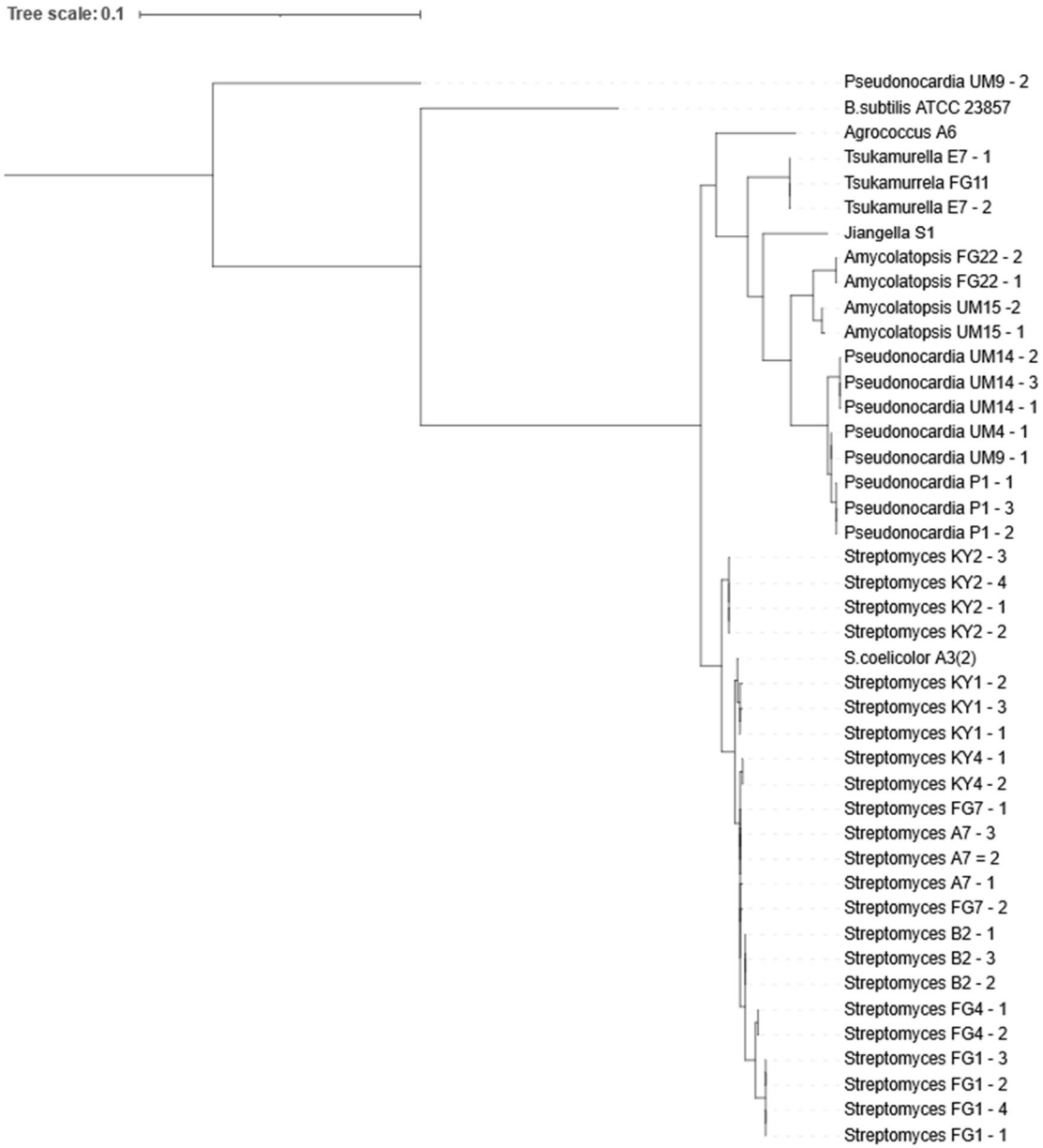


Figure 33 – Phylogenetic analysis of the 18 cultivatable strains. 16S rRNA was extracted from the whole genome sequence using RNAmmer v1.2. Where multiple 16S rRNA sequences were identified, all were included. Alignment was performed using SSU-ALIGN, and the tree was built using GTR with Gamma, all within SILVA. The tree was visualised in the Interactive Tree of Life 6.5.8. *B. subtilis* ATCC 23857 was selected as the outgroup. The labels here contain the strain designation and which unique 16S rRNA they represent; for example, the branches for *Streptomyces* KY1 – 1 and *Streptomyces* KY1 – 2 refer to two unique 16S rRNA sequences both encoded within *Streptomyces* KY1.

Most 16S rRNA gene sequences clustered together by genus, as would be expected, with 16S rRNA gene sequences from the same organism most closely related to each other over those from other strains. For these strains, the 16S rRNA gene sequence encoded the greatest number of times was used for subsequent phylogenetic analysis. If multiple sequences were represented an equal number of times, the sequence closest to the centre of the genome was chosen. A notable exception, however, was *Pseudonocardia* UM9 – 2. This sequence was deemed less related to the other actinomycetes analysed than the outgroup. When this sequence was analysed using NCBI BLAST, it had the highest similarity to the 16S rRNA gene sequence from an uncultured *Sphingomonadaceae* species isolated from an oil spill. Other sequences deemed to have high similarity included the 16S rRNA gene sequence encoded by *Sphingorhabdus sp.* C69, isolated from soil in Vietnam - although this sequence is presented without an associated paper (Nguyen, 2020; Woo et al., 2021). A summary of the most significant alignments is presented in Table 33. None of these had a close relationship to any actinomycete, so *Pseudonocardia* UM9 – 2 was removed from the analysis.

Table 33 - Summary of the results of a BLASTn analysis of the 16S rRNA sequence *Pseudonocardia* UM9 – 2. This analysis showed that this rRNA sequence was more related to Sphingomonadaceae than Pseudonocardiaceae species

Description	Scientific Name	Query Cover	E value	Identity (%)	Accession
Uncultured *Sphingomonadaceae* bacterium clone 4.6m31 16S ribosomal RNA gene, partial sequence	uncultured *Sphingomonadaceae* bacterium	98%	0	97.66%	JN679141.1
Sphingorhabdus sp. strain C69 16S ribosomal RNA gene, partial sequence	*Sphingorhabdus sp.*	96%	0	97.75%	MT756070.1
Uncultured bacterium clone NN77 16S ribosomal RNA gene, partial sequence	uncultured bacterium	100%	0	96.41%	JN869103.1
Uncultured bacterium clone BD07370 16S ribosomal RNA gene, partial sequence	uncultured bacterium	88%	0	99.92%	JQ191144.1
Sphingobium sp. JCM 28248 gene for 16S ribosomal RNA, partial sequence	*Sphingobium sp.* JCM 28248	99%	0	95.87%	LC133595.1

The updated phylogenetic tree, containing each strain only once, is shown in Figure 34. *Streptomyces* KY2 had a closer relationship to the other *Streptomyces* strains in this study than the rare actinomycetes; however, it does stand alone on a separate branch of the tree from the other *Streptomyces* species. The two *Amycolatopsis* strains – UM15 and FG22 – also had a more significant gap between them than expected. The bootstrap value for the branch between the *Amycolatopsis* and *Pseudonocardia* cluster and the other actinomycetes is low (0.548), offering only weak support for this branch. This may be due to the limitations of using 16S gene sequences for the alignment, which can lead to low resolution between species if genes have been transferred horizontally. Further, given the fact that multiple 16S gene sequences were identified in most of the strains, it may that that if different sequences had been selected the resolution of the phylogenetic tree would be improved. It is also likely, given the antiSMASH analysis conducted here, that the genome of these strains outside of the 16S gene sequence, even if the 16S gene sequence is highly similar – resulting is significantly different phenotypes than may be expected from a phylogenetic tree constructed from 16S gene sequences alone.

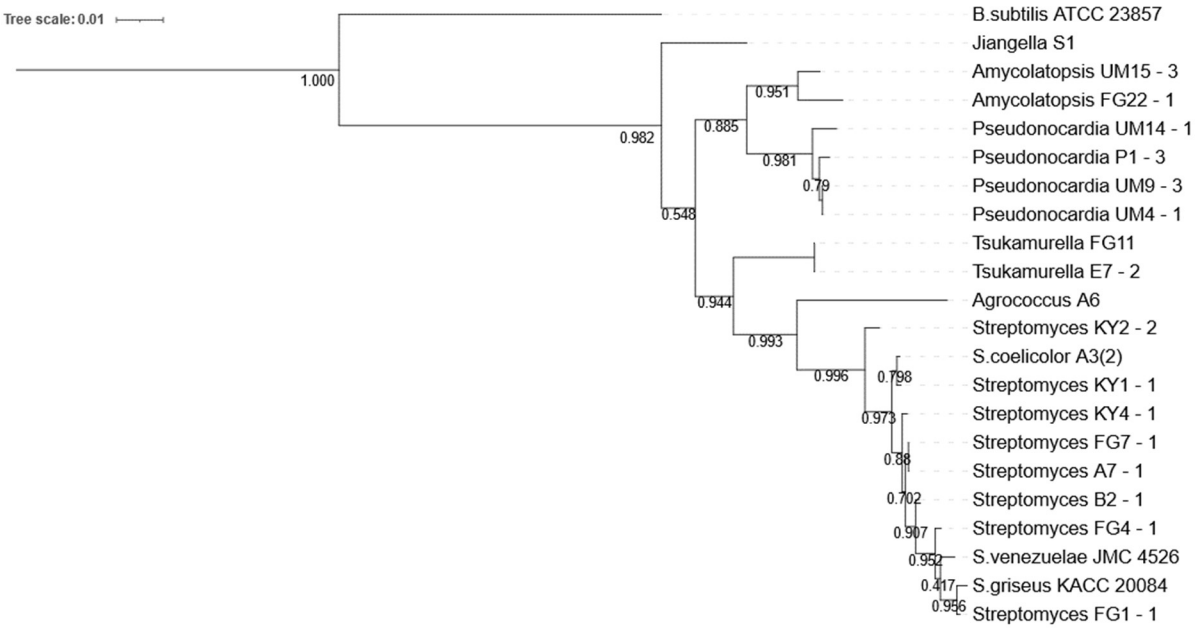


Figure 34 - Phylogenetic analysis of the 18 cultivatable strains, limited to a single 16s rRNA sequence per strain. Alignment was performed using SSU-ALIGN, and the tree was built using GTR with Gamma, all within SILVA. The tree was visualised using the Interactive Tree of Life 6.5.8. *B. subtilis* ATCC 23857 was selected as the outgroup. The different actinomycete species are clustered, although sometimes with low bootstrap values within species. Of note is the low bootstrap value at the branch between the *Amycolatopsis* and *Pseudonocardia* clusters and the other actinomycetes.

3.5 Discussion

This chapter analysed the high-quality full genome sequences of 18 ant-associated actinomycetes for their biosynthetic potential. SEM images of all 18 strains were taken to ensure the strains were sporulating on SFM agar. The SEM images presented here of the rare actinomycetes *Tsukamurella*, *Jiangella* and *Agrococcus,* are believed to be the first reported SEM images of spores of these strains. Select human pathogens were used to challenge the 18 actinomycetes, and their antimicrobial activities across 35 media were compared to the smBGCs predicted in their genomes. A total of 404 smBGCs were predicted to be encoded across the genomes, with only 88 (21.7%) having significant homology to previously reported BGCs, demonstrating the potential of actinomycetes isolated from under-explored environments to be sources of novel chemistry. Additionally, although the rare actinomycetes studied here had fewer smBGCs encoded within their genomes compared to *Streptomyces* species, a higher proportion of these had low homology to previously reported smBGCs. This suggests that rare actinomycetes remain reservoirs of unexplored biosynthetic potential. Further analysis of these genome could be conducted using tools such as Biosynthetic Gene

Similarity Clustering and Prospecting Engine (BiG-SCAPE). BiG-SCAPE would allow comparison of the smBGCs across the genomes studied here, potentially identifying smBGCs shared across strains exhibiting antimicrobial activity – which may indicate that smBGC is more likely to encode the antimicrobial compound. This information may also be interesting to compare to the phylogenetic analysis to determine if any smBGCs are clustered within genera.

By performing bioassays on 35 distinct media, antimicrobial activity was elicited from all 18 strains, whilst only six strains possessed any antimicrobial activity on SFM – the default medium for many actinomycetes experiments in laboratory conditions. Further, ISP2 and ISP4 only elicited activity on three and two strains respectively, demonstrating their relative inability to elicit antimicrobial production from the strains studied here. This ability for non-conventional media to elicit antimicrobial activity demonstrates how pleiotropic techniques can still be used to identify strains producing potentially novel antimicrobial compounds. Those conducting future attempts at eliciting antimicrobial activity from actinomycetes in a manner similar to that used here should be aware that traditional media may not be the most efficient at eliciting antimicrobial activity. FML and SM25 proved the most effective media to elicit antimicrobial activity among the strains tested here, with GYM+10µg/mL streptomycin proving most likely to elicit activity against *E. coli*. Given the multiple combinations of stress factors different media ingredients can inflict on an actinomycete strain, further work would be required to identify optimal carbon and nitrogen sources for eliciting activity – although these may vary as other conditions, such as pH or metal ion concentration, also change. FML contains both a simple sugar, glucose, and a complex carbon source, peptone, in addition to a complex nitrogen source, malt extract. It may be that the glucose in the media allows for rapid cell replication, and once depleted, the more complex carbon source triggers the upregulation of otherwise cryptic smBGCs. This combination may also more closely mirror the environment on the ant carapace, where the bacteria are provided with a carbon source by the ant – which would potentially increase antimicrobial expression by the strains studied here, given their ecological niche. SM15 proved the least effective at eliciting antimicrobial activity, with many strains not able to survive upon it. There is no media component unique to SM15 among the media used in this study – SM7 contains MOPS, Cas-amino acids were proven to have minimal effect on antimicrobial expression when added to SFM and glycerol solution is also present in SM25 and SM32. SM7, SM25 and SM32 all had a higher survival rate

than SM15. It may be that the relatively high concentrations of metal ions impacted the ability for actinomycetes to grow. Although SM7 contained potassium, calcium and sodium concentrations similar to SM15, SM7 did not contain EDTA – a metal chelator that may make much of these metal ions inaccessible to the actinomycetes. It is therefore plausible that lack of iron and calcium inhibited the ability for many strains to grow. Further work could be performed to optimise growth conditions for the rare actinomycetes – given the high time to sporulation of many of these strains optimising their cultivation may allow for greater ease when performing future experiments. However, this may also alter the expression of any encoded antimicrobials, so an optimised media would only be useful for some types of experiments, such as genetic modification attempts. These investigations may also improve the understanding of these rare actinomycetes, which are under-studied compared to *Streptomyces* species, especially their global regulation.

Also in this chapter, the antimicrobial properties of the strains were compared to their antiSMASH predicted smBGCs. Most of the strains do not have smBGCs with high homology to previously reported antimicrobial encoding smBGCs suggesting at least some of these strains could provide sources of antimicrobials with novel mechanisms of action that evade current resistance mechanisms. It is worth noting, however, that this analysis was limited to NRPS, PKS and RiPP encoding smBGCs. There is a possibility that these strains possessed an antimicrobial encoded by a terpene-encoding smBGC, like, for example clorobiocin produced by *S. roseochromogenes* (O'Neill, 2017). Later in this thesis, a selection of these strains with antimicrobial activity but no smBGCs that could adequately explain this activity will be further investigated.

Finally, a phylogenetic analysis was conducted using the 16S gene sequences of the 18 actinobacteria. This showed the different species in this study clustering including with the model organisms they were compared with, as would be expected. Less expected is the relatively low bootstrap values of some branches, which may be due the limitations of using 16S gene sequences rather than whole genomes to construct the phylogenetic tree. It would have been preferable to have utilised a tool such as autoMLST to generate the phylogenetic tree over those used here. AutoMLST generates Multi-Locus Species Trees (MLSTs) to produce phylogenetic trees, aligning several regions across the genome to build a phylogenetic tree using its own version of FindModel to identify the optimal tree building model. This allows for

a more comprehensive look at the relatedness of the different strains and provides all of the tools required to produce and analyse a phylogenetic tree in one place .

4 Extraction and Fractionation of Antimicrobials from Selected Actinomycete Strains

4.1 Identification of Potentially Novel Antimicrobials

In Section 3, 18 actinomycete strains isolated from tropical fungus-growing ant colonies were assessed for their ability to inhibit *B. subtilis, E.coli* and *C. albicans* across a range of growth media. The observed antimicrobial activity was then compared to the predicted products of smBGCs. It was hypothesised that strains demonstrating antimicrobial activity but were predicted not to contain a smBGC encoding a known antimicrobial were potentially producing a novel antimicrobial. For *Streptomyces* FG4, *Streptomyces* KY2, *Streptomyces* B2, *Streptomyces* A7, *Streptomyces* FG1, *Amycolatopsis* UM15 and *Amycolatopsis* FG22 there was no smBGC with high homology to previously-reported smBGCs encoding antimicrobials – in this chapter, extractions of these unexplained antimicrobials were attempted from. This was performed with the aim of extracting, and then identifying, antimicrobials with a potentially novel mechanism of action that may go on to be useful in clinical settings.

Solvent extractions were conducted using dH_2O, methanol and ethyl acetate. Given the abundance of water in the environment, dH_2O was chosen as it is the most likely solvent the compound would be in solution within the natural environment. Methanol and ethyl acetate are solvents in which many substances can be dissolved or suspended, with methanol having a high polarity for more hydrophilic compounds and ethyl acetate with low polarity for more lipophilic compounds. Additionally, freeze-thaw extractions were attempted. During freezing, ice crystals inside the actinomycete cell and media expand, causing cell lysis and splitting of the media. This can release compounds trapped within, which can then be extracted once the ice has melted. By combining all of the solvent extractions with freeze-thaw extracts, at least one method will likely yield an extract that possesses antimicrobial activity, assuming that activity originates from an antibiotic. The antimicrobial activity caused by siderophores and enzymes is not likely to be extracted using these methods. Antimicrobial enzymes, such as lactoperoxidase, lysozyme and lysostaphin, can inhibit cell growth directly or interfere with biofilm formation by degrading proteins, degrading polysaccharides or causing oxidative stress (Thallinger *et al.*, 2013). Previous studies of *Streptomyces* species have revealed that the chitinases produced by some strains can hydrolyse the cell walls of plant pathogenic fungi,

inhibiting fungal growth (Anitha and Rabeeth, 2020). For enzymes to be extracted, methods that minimise the risk of denaturation have to be used in place of solvent extractions, for example, using sonication or French press to lyse cells. On the other hand, Siderophores can be extracted by centrifuging liquid cultures and then partially purified using chromatographic techniques such as ion exchange chromatography and solid-phase extraction (Koppisch *et al.*, 2005; Sayyed and Chincholkar, 2006). This can still result in samples comprising a mixture of compounds, and false positives are not uncommon, which has led to the development of titanium nanoparticle purification methods (Egbers *et al.*, 2020). The lack of antimicrobial activity observed in the 18 strains studied here when cultivated in liquid medium limited the ability for siderophores to be isolated – and given the limited usefulness of siderophores in clinics, they are of less interest to this study.

4.2 Optimisation of Extraction Techniques

Before purifying the antimicrobial compounds, they needed to be extracted from the media upon which the actinomycete was cultivated. Optimisation of this process took some time before the method described in Section 2.17 was utilised. Initial extractions were proceeded by first cultivating the strain on all the different growth media upon which it had demonstrated activity for the time described in Section 3.1. These growth media were sliced using a razor blade into pieces of approximately 0.5cm^2. For freeze/thaw extractions, these pieces were then placed in a large glass beaker at -20°C overnight to allow cell membranes to be broken by ice crystals and release any chemicals inside. This also causes ice crystals to disrupt the structure of the medium, breaking it apart and releasing the water inside once the medium is thawed – hopefully with the compounds of interest in solution. For solvent-based extractions, the sliced-up media were submerged in the selected solvent – dH_2O, methanol or ethyl acetate, overnight. In both freeze/thaw and solvent extraction, the resulting solvent was filtered and dried down in an SP Scientific Genevac EZ-2 Elite before being resuspended in the minimal amount of the same solvent used during the extraction to dissolve all the material collected. The resulting solution was tested for its antimicrobial properties at three different dilutions (1x, 0.1x and 0.01x) as per Section 2.22. If all three dilutions demonstrated a similar ability to inhibit microbial growth, it was deemed likely that this activity was caused by siderophore activity sequestering iron rather than an antimicrobial. This initial method resulted in the extraction of metabolites with antimicrobial activity from *Streptomyces* FG1

grown on FML against *B. subtilis* and *C. albicans*, using methanol and ethyl acetate, and *Amycolatopsis* UM15 grown on FML against *B. subtilis* and *C. albicans,* using just ethyl acetate. No other conditions resulted in extracts exhibiting antimicrobial activity, except for extractions from *Amycolatopsis* FG22. When ethyl acetate extractions of *Amycolatopsis* FG22, cultivated on SFM, SM7 and SM15, were tested for antimicrobial properties, all three dilutions of the extract showed similar ability to inhibit *C. albicans* but demonstrated no activity against *B. subtilis* or *E. coli*. This, combined with the lack of antifungal activity exhibited by *Amycolatopsis* FG22 described in Section 2.6, led to the conclusion that it was likely siderophore activity causing the antimicrobial activity, so no further extractions from this strain were attempted.

In an attempt to extract antimicrobials from a wider variety of actinomycetes, the extraction method was optimised. Initial optimisation included placing the extracts during the soaking stage onto a reciprocating shaker at 120rpm and wrapping the tubes in blue roll, as shown in Figure 35. This was intended to improve extraction from the media by using the shaker to increase diffusion and the blue roll to protect the extract from being degraded by UV light. This did not noticeably affect the extractions performed, but it was decided to maintain both these changes for future extractions regardless. Further optimisation was performed when removing debris from the extract. It was speculated that the use of filter paper may have contributed to the lack of activity if the active product was bound to the filter paper. Extracts were thus performed without filter paper; instead, the crude extract was centrifuged at 4,000rpm for 10 minutes to remove debris, and the supernatant was poured off for further processing. By replacing the filter paper with centrifugation, antimicrobial compounds were extracted from *Streptomyces* FG4 on SM6 and FML against *B. subtilis* using ethyl acetate, in addition to the antimicrobial compounds previously extracted from *Streptomyces* FG1 and *Amycolatopsis* UM15. Further optimisation was then performed at the soaking stage of the extraction. The soaking time was reduced from overnight to two hours in the event the antimicrobial was degrading overnight, but this did not affect the extractions that had thus far not demonstrated antimicrobial activity. No extractions of *Streptomyces* KY2*,* B2 or A7 demonstrated antimicrobial activity using this method. This optimisation resulted in the extraction method described in Section 2.17, which is used for all the extracts described here

unless stated otherwise. Despite multiple optimisation attempts, no freeze/thaw extractions proved able to inhibit the growth of the microorganisms tested.

Figure 35 - Extractions being performed in Durans, with (top) and without (bottom) blue-roll to protect the contents from UV light. Flasks were wrapped in blue roll to protect the same from UV light and placed on a lateral shaker to increase the compound's diffusion rate out of the media.

Extractions were next attempted for *Streptomyces* KY2, B2 and A7 when challenged by the pathogens they were able to inhibit during the bioassays performed in Section 3.2, to test if the presence of the hostile bacteria or fungi was required to elicit production of the antimicrobial. For these extraction attempts, spores of the actinomycete were placed into the

centre of a 90mm petri dish and incubated for the time described in Section 3.1. These were then overlaid with soft LB inoculated with the pathogen of interest in the same manner described for bioassays in Section 2.6. The area of medium containing the actinomycete colony and the zone of inhibition was then cut out and removed before being chopped and extracted as per Section 2.17. This also proved unsuccessful at extracting antimicrobial activity (not shown). It was, therefore, decided to progress with only the extractions from *Streptomyces* FG4 and FG1 and *Amycolatopsis* UM15, given their relative ease of extraction. For these strains, the media which produced the highest mass of bioactive extract was used.

The extracts generated throughout this chapter were not standardised and may have varied in concentration. This limits the ability to compare the antimicrobial activity of an extract between strains and between media. Only replicated within one strain and medium combination can be compared with each other, as replicates were standardised.

4.2.1 *Streptomyces* FG4 extractions

As presented in Table 34, *Streptomyces* FG4 extractions were performed on Min, SM12 and Min+NAG – the media upon which the bioassays described in Section 3.3.7 showed *Streptomyces* FG4 was able to inhibit *B. subtilis, E. coli* and *C. albicans.* Further, extractions were performed on *Streptomyces* FG4 colonies cultivated on SFM and FML, with inhibition of both *B. subtilis* and *C. albicans* observed when these extractions utilised ethyl acetate or methanol. Extractions from SM6 using ethyl acetate demonstrated the ability to inhibit *B. subtilis*, *E. coli* and *C. albicans* but methanol extracts of SM6 showed no such abilities. Other growth media where only one organism was inhibited were deprioritised, and extractions were not performed. Additionally, cultures using GYM + 10µg/mL streptomycin were not extracted as there were concerns that the streptomycin could be extracted and give a false-positive result. Although this risk could have been designed out of the experiment by placing a mock-inoculated plate in the incubator next to the inoculated plates, the fact that bioactivity was successfully extracted from other growth media made this unnecessary.

Table 34 - Yield and Bioactivity of Extractions of *Streptomyces* FG4 Colonies. Ethyl acetate resulted in higher yield extract from all media other than minimal. Compounds able to inhibit *B. subtilis* were extracted across the widest variety of media (4), with the ethyl acetate extraction of *Streptomyces* FG4 cultivated on FML being the only extract able to inhibit all three indicator strains.

			Activity Observed Against		
Growth Medium	**Extraction Solvent**	**Yield (mg of extract per L of media)**	***B. subtilis***	***E. coli***	***C. albicans***
SFM	Ethyl Acetate	23.8	N	N	N
	Methanol	4.6	N	N	N
FML	Ethyl Acetate	46.4	Y	N	Y
	Methanol	38.5	Y	N	N
Min	Ethyl Acetate	41.3	N	N	N
	Methanol	42.0	N	N	N
SM6	Ethyl Acetate	21.4	Y	Y	Y
	Methanol	16.8	N	N	N
SM12	Ethyl Acetate	6.8	Y	N	N
	Methanol	12.1	N	N	N
MinNAG	Ethyl Acetate	14.9	N	N	N
	Methanol	17.6	N	N	N

The compound produced by *Streptomyces* FG4 responsible for bioactivity against *B. subtilis* was extracted from FML, SM6 and SM12, whilst on FML, *Streptomyces* FG4 was active against *E. coli* and the compound responsible for this activity was also extracted. Extracts from *Streptomyces* FG4 grown on SM6 were also active against *E. coli* and *C. albicans* but not *B. subtilis,* as shown in Figure 36. Of these active extractions, only the FML extraction against *B. subtilis* was performed using methanol; all other bioactive extractions were performed using ethyl acetate, which also produced higher yields from SFM, FML and SM6. Methanol did provide a higher yield in extractions from Min, SM12 and MinNAG, despite not producing an active extract on SM12 when ethyl acetate did.

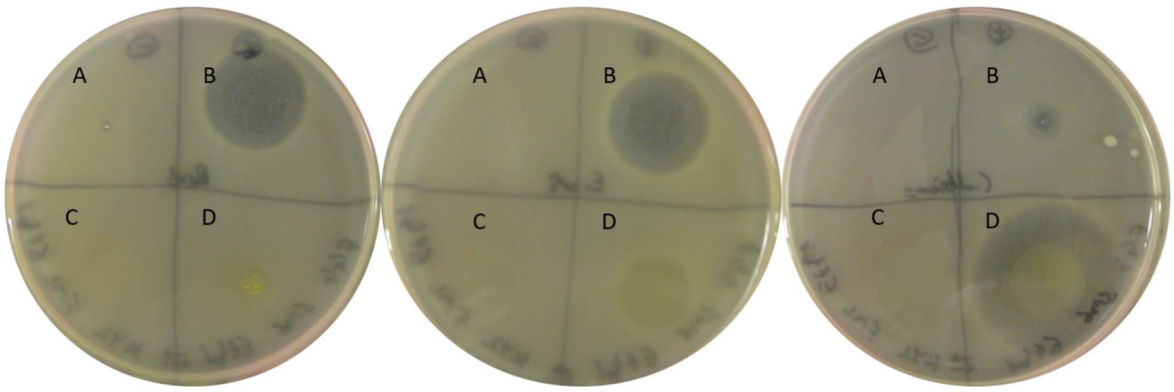


Figure 36 – Bioassays of extracts of *Streptomyces* KY2 and FG4 when challenged by *B. subtilis* (left), *E. coli* (centre) and *C. albicans* (right). A; negative control of 80/20 acetonitrile/dH_2O, B; positive control of apramycin (*B. subtilis*), kanamycin (*E. coli*) or nystatin (*C. albicans*), C; extract of KY2 cultivated on FML, D; extract of FG4 cultivated on SM6.

4.2.2 *Streptomyces* B2 extractions

For *Streptomyces* B2, extractions were performed after cultivation on SM6 with *B. subtilis* or *C. albicans,* although none of these proved bioactive, as presented in Table 35. The yields were calculated by weighing the section of media inside the zone of inhibition, including the *Streptomyces* B2 colony, rather than using the total volume of medium poured. Due to this requirement for co-culturing to elicit antimicrobial activity when other strains did not require this, it was decided not to progress with the purification of the extract from *Streptomyces* B2.

Table 35 - Yield and Bioactivity of Extractions of *Streptomyces* B2 Colonies. Ethyl acetate extractions resulted in higher yields when *Streptomyces* B2 was cultivated alone or with *B> subtilis*, but methanol extract had a higher yield when challenged by *C. albicans*. The only condition able to inhibit any indicator strain, was when co-culturing with *B. subtilis*, which was able to inhibit *B. subtilis.*

			Activity Observed Against		
Growth Medium	**Extraction Solvent**	**Yield (mg of extract per L of media)**	***B. subtilis***	***E. coli***	***C. albicans***
SM6	Ethyl Acetate	26.6	N	N	N
	Methanol	17.2	N	N	N
SM6 (challenged with *B. subtilis)*	Ethyl Acetate	16.4	Y	N	N
	Methanol	14.9	Y	N	N
SM6 (challenged with *C. albicans)*	Ethyl Acetate	14.1	N	N	N
	Methanol	16.0	N	N	N

4.2.3 *Streptomyces* A7 extractions

Extractions of the antimicrobial(s) produced by *Streptomyces* A7 were performed on SFM, SPY, MYM, SM7 and SM20, and GYM + 10µg/mL streptomycin. Upon all these growth media, *Streptomyces* A7 demonstrated a large (>10mm) zone of inhibition when challenged by *B. subtilis*, except on GYM + 10µg/mL streptomycin, where a smaller zone of inhibition was observed, but inhibition of *E. coli* and *C. albicans* was observed. As previously discussed, extractions from a GYM + 10µg/mL streptomycin plate required a blank number of plates totalling an equivalent volume of media to be extracted to ensure any extract activity observed was due to products from the *Streptomyces* A7 colony and not the streptomycin contained within the media. The only condition in which extracts from *Streptomyces* A7 could inhibit *B. subtilis* was when challenged by *B. subtilis* on GYM + 10µg/mL streptomycin.

Table 36 presents the activity and yields of the extractions performed on *Streptomyces* A7 colonies. The yields for the extractions performed when indicator strains challenged the strain were calculated by weighing the section of media inside the zone of inhibition, including the *Streptomyces* B2 colony, rather than using the total volume of media poured. Due to the challenge involved in these extractions, and the extra work and waste caused by needing to rule-out streptomycin activity, it was decided not to progress with the purification of the antimicrobial produced by *Streptomyces* A7.

Table 36 - Yield and Bioactivity of Extractions of *Streptomyces* A7 Colonies. Only *B. subtilis* was inhibited by either extract, with ethyl acetate producing a slightly higher yield.

			Activity Observed Against		
Growth Medium	**Extraction Solvent**	**Yield (mg of extract per L of media)**	***B. subtilis***	***E. coli***	***C. albicans***
GYM + 10µg/mL streptomycin (when challenged by *B. subtills*)	Ethyl Acetate	12.6	Y	N	N
	Methanol	10.8	Y	N	N

4.2.4 *Streptomyces* FG1 extractions

Extractions of *Streptomyces* FG1 were performed on FML, YEME, SM20 and GYM + 10µg/mL streptomycin, presented in Table 37. The antimicrobial responsible for inhibiting *B. subtilis* was relatively easy to extract, present in five of the eight extraction conditions. However, the anti-*C. albicans* activity could only be extracted when *Streptomyces* FG1 was grown on FML, and extractions were performed using ethyl acetate as a solvent. No activity could be extracted from colonies grown on GYM + 10µg/mL streptomycin despite yields of similar mass to other growth media. Unfortunately, the anti-*E. coli* activity could not be extracted. This is especially disappointing when you consider that *Streptomyces* FG4 inhibited kanamycin-resistant *E. coli.* Due to activity against *C. albicans* only being observed on extractions from

FML, it was decided that this would be the medium of choice for further extractions of *Streptomyces* FG1.

Table 37 - Yield and Bioactivity of Extractions of *Streptomyces* FG1 Colonies. *B. subtilis* was inhibited by all ethyl acetate extractions, except when GYM + 10µg/mL streptomycin was used. *C. albicans* was only inhibited when *Streptomyces* FG1 was cultivated on FML and the extract used ethyl acetate. No extract was able to inhibit *E. coli*.

			Activity Observed Against		
Growth Medium	**Extraction Solvent**	**Yield (mg of extract per L of media)**	***B. subtilis***	***E. coli***	***C. albicans***
FML	Ethyl Acetate	36.8	Y	N	Y
	Methanol	32.4	Y	N	N
YEME	Ethyl Acetate	24.1	Y	N	N
	Methanol	26.3	N	N	N
SM20	Ethyl Acetate	28.2	Y	N	N
	Methanol	31.6	Y	N	N
GYM + 10µg/mL streptomycin	Ethyl Acetate	31.2	N	N	N
	Methanol	27.7	N	N	N

4.2.5 *Amycolatopsis* UM15 extractions

As presented in

Table 38, extractions from *Amycolatopsis* UM15 cultivated on FML were able to inhibit *B. subtilis* and *C. albicans* activity. No other extraction condition was able to inhibit *C. albicans,* and none were able to inhibit *E. coli*, despite *Amycolatopsis* UM15 showing the ability to do so in bioassays. Ethyl acetate proved more likely to result in a bioactive extraction, with extracts from all media used resulting in activity against *B. subtilis* and methanol only producing bioactivity from YEME. Given that *Amycolatopsis* UM15 is a rare actinomycete and the relative ease of extracting the antifungal activity when grown on FML, it was decided to attempt purification of the antimicrobial(s) produced that may be responsible for the observed activity.

Table 38 - Yield and Bioactivity of Extractions of *Amycolatopsis* UM15 Colonies. All ethyl acetate extractions proved able to inhibit *B. subtilis*, although no extractions were able to inhibit *E, coli*.

			Activity Observed Against		
Growth Medium	**Extraction Solvent**	**Yield (mg of extract per L of media)**	***B. subtilis***	***E. coli***	***C. albicans***
SFMNAG	Ethyl Acetate	24.6	Y	N	N
	Methanol	27.1	N	N	N
FML	Ethyl Acetate	28.6	Y	N	Y

	Methanol	21.9	N	N	N
YEME	Ethyl Acetate	34.1	Y	N	N
	Methanol	27.5	Y	N	N
MinNAG	Ethyl Acetate	12.9	Y	N	N
	Methanol	13.6	N	N	N

4.3 Purification of Bioactive Extracts

Bioassay-guided fractionation (BGF) was performed on bioactive extracts from *Streptomyces* FG4, FG1 and *Amycolatopsis* UM15. To perform a BGF, the sample is run through a chromatography column, such as a C-18 HPLC column, with the eluent from the column collected and separated into a number of separate containers, either when a noticeable peak is detected using a spectrophotometer, for example, a UV or mass spectrometer, or after a period of time has passed. These fractions are tested for biological activity, as described in Sections 2.22 and 2.23. The spectroscopy results from active fractions are then further analysed using LCMS to determine if any peaks can explain the biological activity or, if there are multiple peaks, the process is repeated in an attempt to ensure each peak is isolated from the others – ideally resulting in a pure sample that exhibits biological activity. BGF is an effective method of purifying biologically active compounds, but it is resource intensive in terms of human and machine time, as the process requires extensive use of HPLC and evaporators. Changes in the concentration of the active compound when testing fractions may obscure any activity, particularly if the product is diluted. Thus, when performing a BGF, it is important to maintain the concentration of the product throughout. For example, if 500µL of extract is injected into the first fractionation run, all bioactivity tests must also have the fraction dissolved in 500µL of solvent. It is often also advisable to test the crude extract alongside the fractions in case loss of activity can be explained by the compound degrading

over time or by exposure to oxygen or UV light. Extractions from *Streptomyces* FG4 grown on SM6, *Streptomyces* FG1 grown on FML, and *Amycolatopsis* UM15 grown on FML, all using ethyl acetate, were selected for BGF as per Section 2.19. Additionally, *Streptomyces* FG4 cultivated on SM6 and extracted using methanol was also used for BGF.

4.3.1 Analysis of *Streptomyces* FG4 Extracts

Prior to fractionation, an analytical LC/MS analysis of two ethyl acetate extractions performed from *Streptomyces* FG4 cultivated on SM6 was performed, as shown in Figure 37 and Figure 38, referred to as extracts A and B, respectively. Table 39 summarises the 15 major peaks observed on the UV, evaporative light scattering detector (ELSD) and mass spectrometry detectors for the two extracts. Both extractions presented highly similar chromatograms, with peaks eluting at similar time points, including the largest single peak, according to UV and ELSD detection, at 4.067 and 4.068 minutes. Similarly, a large peak on the UV detector was recorded at 6.040 and 6.021, in both cases demonstrating absorption at 210, 220 and 280nm. Neither sample possessed a peak absent in the other. None of the peaks identified could be attributed to known antimicrobials, so LC/MS/MS was performed as per Section 2.21 and analysed using Compound Discoverer 3.1. This revealed that the peak with a retention time of 4.63 minutes was potentially cyclo(leucylprolyl), an antifungal pyrrole-class compound (Bajpai *et al.*, 2018). There is some uncertainty in this prediction, as the positive ion MS lacks a fragment at 125.1m/z, the highest abundance fragment detected in the study discovering the compound. The originating study did perform gas-chromatography/MS rather than the LC/MS, which may help to explain the difference in fragments observed, however (Bajpai *et al.*, 2018). No other peak observed had any similarity to previously-reported compounds.

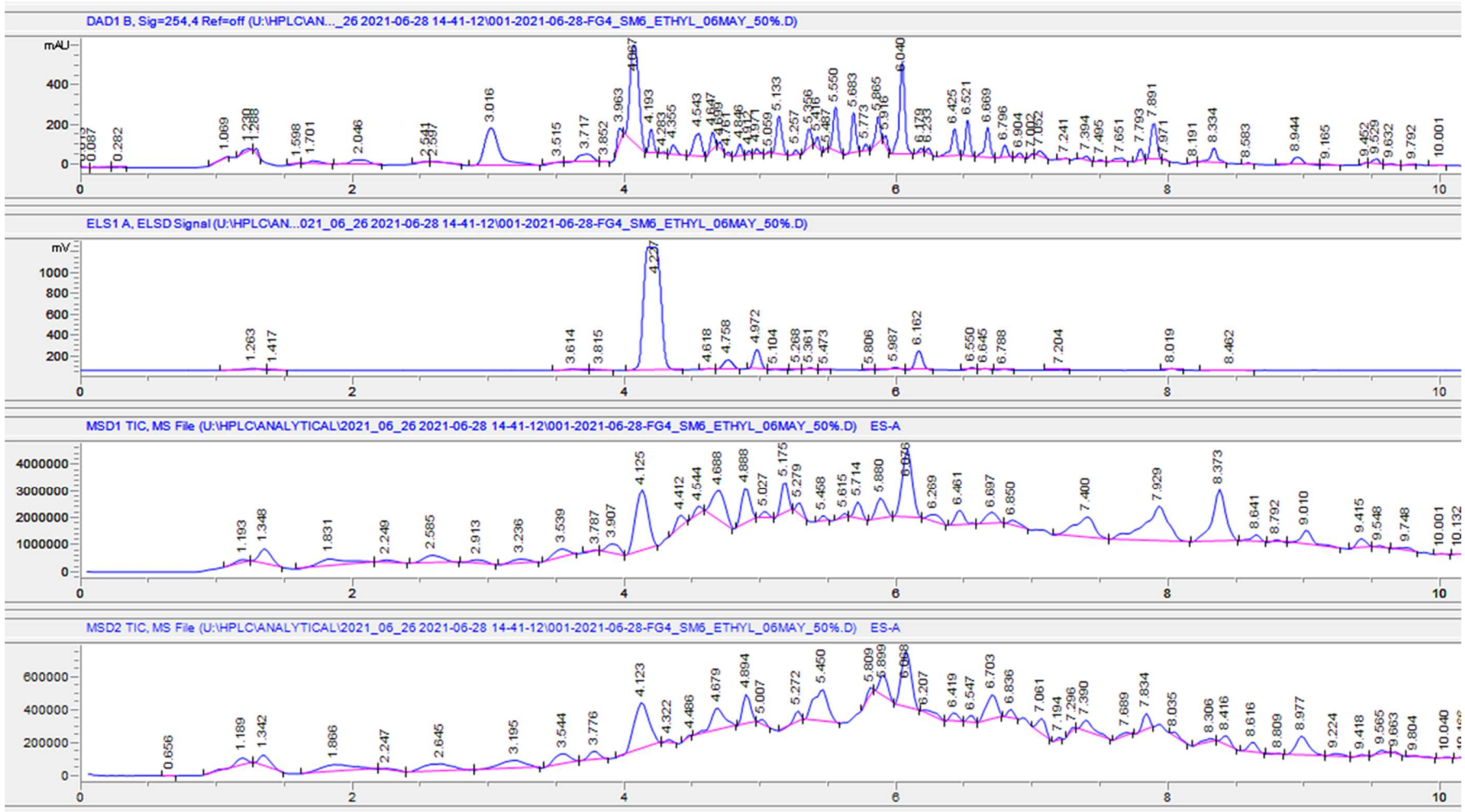


Figure 37 – Analytical LC/MS of the Ethyl Acetate Extract of *Streptomyces* FG4 Cultivated on FML, Extract A, with chromatograms from a (top) UV spectrometer at 254nm, (second from top) ELSD, (second from bottom) positive ionisation mass spectrometry and (bottom) negative ionisation mass spectrometry.

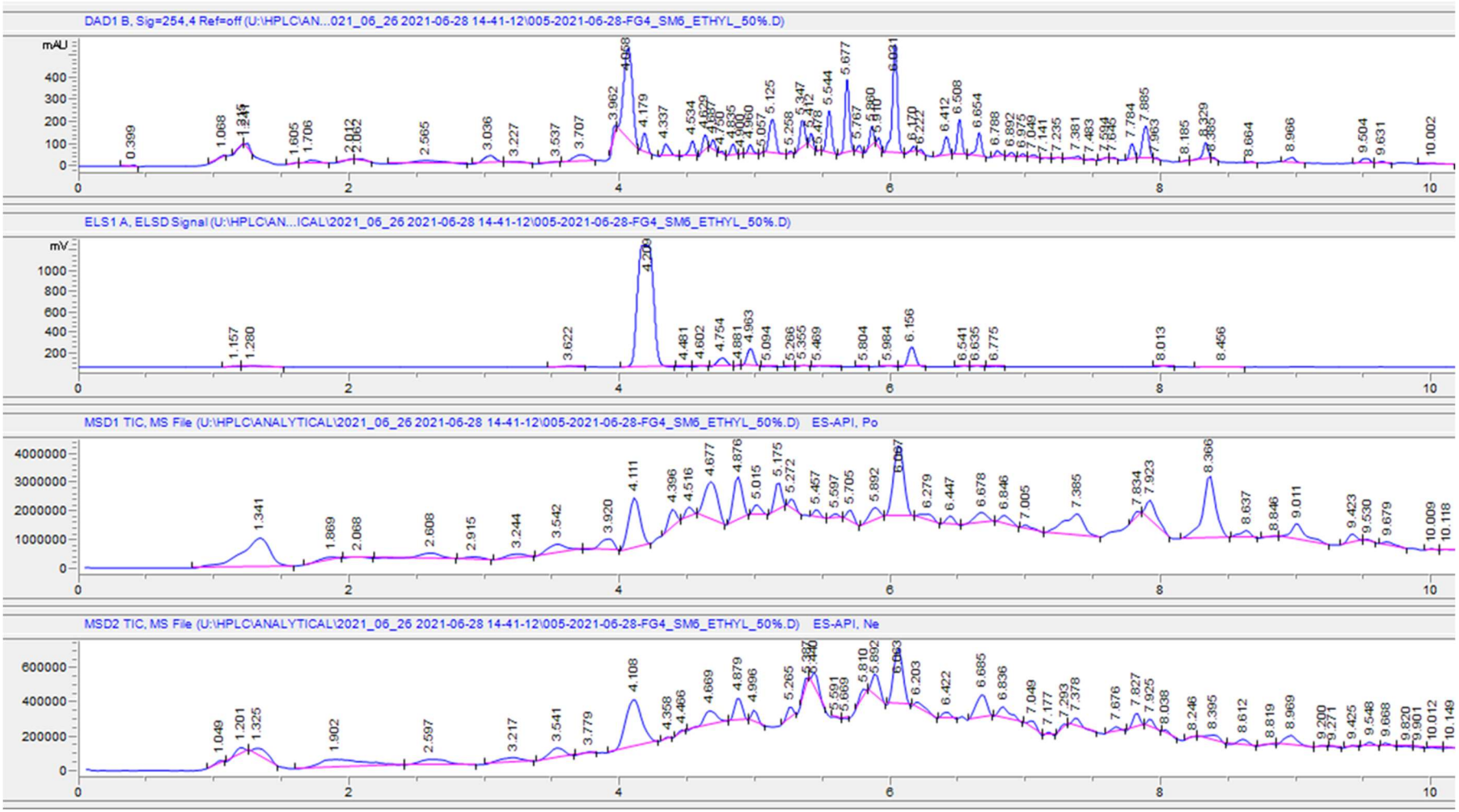


Figure 38 – Analytical LC/MS of the Ethyl Acetate Extract of *Streptomyces* FG4 Cultivated on FML, Extract B - with chromatograms from a (top) UV spectrometer at 254nm, (second from top) ELSD, (second from bottom) positive ionisation mass spectrometry and (bottom) negative ionisation mass spectrometry.

Table 39 – Summary of Major Peaks from the LC/MS analysis of both *Streptomyces* FG4 Extracts

Retention Time[1]	UV Absorption (nm)	ELSD (mV)[1]	MS+ (m/z)	MS- (m/z)
1.23	260	50	340	338
1.60	190, 210, 270	0	-	-
3.04	210, 250, 280	0	-	-
4.07	205, 230, 275	1250	493	491
4.63	200, 250	0	211	209
5.13	225, 280	0	245	-
5.35	210, 260, 300	0	273	271
5.55	215, 255, 320	25	-	433
5.67	220, 265	0	480	478
5.86	190, 220, 300	50	405	-
6.03	210, 220, 280	250	203, 246	201, 244
6.20	N/A	0	272	302
6.70	210, 290	25	208, 225	206, 223
7.87	210, 300	0	231	239
8.33	200, 245, 290	50	264	-

1. The values reported here are the average of those observed in extracts A and B.

Given the similarity of the *Streptomyces* FG4 extracts A and B, they were combined for BGF, performed as per Section 2.19. The LC/MS chromatogram, shown in Figure 40, shows far fewer peaks than the analytical runs. This is expected as the ELSE, MS and UV spectrometer are set to less-sensitive settings during preparatory runs to protect them from the larger quantity of sample injected into the instrument. The sample was divided into 29 fractions,

with a new fraction starting every 15 seconds. The three observed peaks, with retention times of 2.549, 2.703 and 3.121 minutes, were split into fractions 4 for the peaks at 2.549 and 2.703 and 5 for the 3.121 peak, as described in Table 40. Each of the 29 fractions was then dried and resuspended in the same volume of solvent initially injected into the LC/MS to maintain concentration. This was followed by testing for biological activity as per Section 2.22, revealing that fractions 23-28 could inhibit *B. subtilis, as* shown in Figure 39 and 24-28 *E. coli.* No fractions were able to inhibit *C. albicans*. None of the fractions containing the three peaks detected during preparatory LC/MS showed any ability to inhibit any indicator strain.

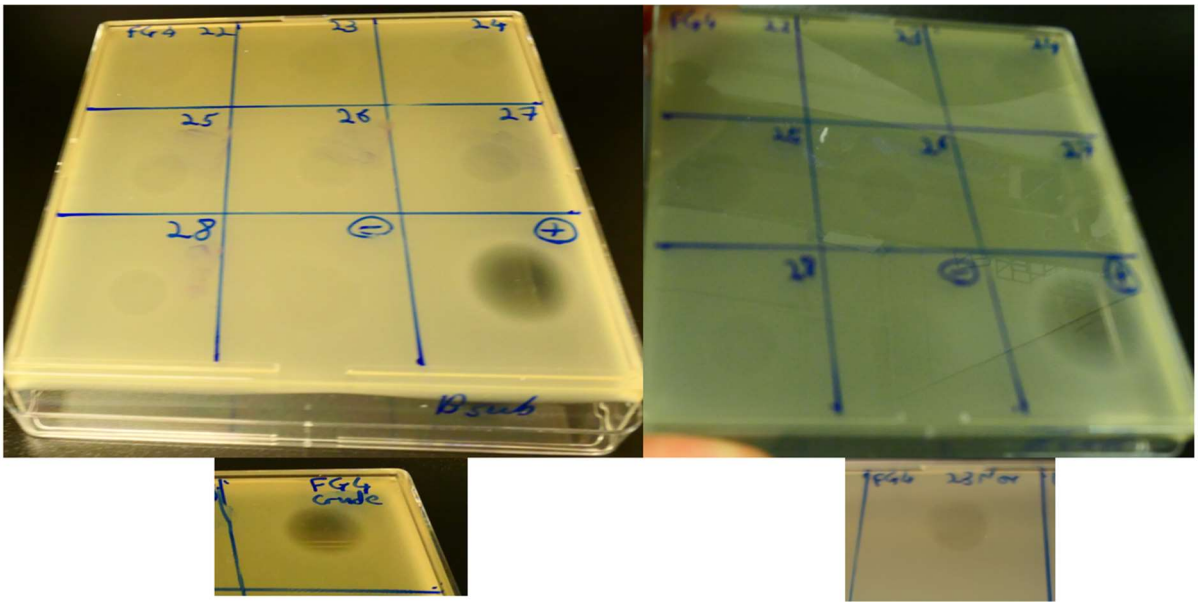


Figure 39 - Activity Assay of Fractions 22-28 of *Streptomyces* FG4 Extract, against (left) *B. subtilis* and (right) *E. coli*. The lower images are of the activity of the crude extract. No fraction showed an ability to inhibit either indicator organism that rivalled the positive control or crude extract.

Figure 40 – Preparatory LC/MS of the Ethyl Acetate Extract of *Streptomyces* FG4 Cultivated on FML - with chromatograms from a (top) UV spectrometer at 254nm, (second from top) ELSD, (second from bottom) positive ionisation mass spectrometry and (bottom) negative ionisation mass spectrometry.

Table 40 – Peaks Detected from the Fractionation of the *Streptomyces* FG4 Extracts

Retention Time	Fraction Number	UV Absorption	[M+]	[M-]
2.549	4	200	-	-
2.703	4	190, 230	257	312
3.121	5	200	493	218

For many samples, there was significant difficulty in determining the activity of the fractions as, in many instances, the zone of clearance was observable but faint. This made imaging the zones of clearance difficult. No fractions demonstrated a zone of inhibition that rivalled the positive control. That the activity is split across a wide number of fractions suggests that the compound responsible for the activity may be getting stuck on the column or is highly lipophilic. Another possibility is that multiple compounds combined are responsible for the activity and eluted at different times; however, the activity was spread across so many adjacent fractions that it does make this less likely. Potentially, *Streptomyces* FG4 encodes an smBGC similar that produces multiple compounds, some of which are intermediates for the final antimicrobial product. These intermediates may also have some antimicrobial activity. This is observed in the formicamycin BGC in *S. formicae*, in that the formicamycin BGC encodes both the final product antimicrobial and an intermediate stage, fasamycin, that possesses (comparatively mild) antimicrobial properties that have a different retention time (Qin *et al.*, 2017b). Alternatively, the smBGC encoding the antimicrobial may be similar to the friulimicin A/B/C/D encoding smBGC in *Actinoplanes friuliensis* – an smBGC that encodes four compounds - in addition to intermediates - which may elute over a broad range of retention times (Schneider *et al.*, 2009). If the BGC in *Streptomyces* FG4 behaves similarly to this, and each of these compounds possesses a mild ability to inhibit microbial growth, then this might explain why multiple fractions demonstrate antimicrobial activity. However, none matches the antimicrobial activity of the original extract. Surprisingly, fractions 23-28 demonstrated the ability to inhibit *B. subtilis* across all repeat experiments when the crude extract did not demonstrate activity, as shown previously in Figure 39. It is not clear why this occurred.

To better visualise the antimicrobial activity of the fractions, bioassays were performed using disk diffusion assays, as per Section 2.23. The disk diffusion assays showed no activity from any of the fractions, with the indicator strains able to grow up to the edge of the disks, as shown in Figure 41. It may be that the disks obscured any evidence of inhibition if that inhibition was mild and the indicator strain was only inhibited directly under the disk. This would suggest that the compound was hydrophobic and did not diffuse readily through the media. Other possibilities may be that the compound was entirely unable to diffuse out of the disk so was not able to interact with the bacteria to inhibit them, or if some were able to leave the disk, it still resulted in a lower concentration due to some of the compound being on the disk instead of diffusing into the media. To better understand if the reduction in antimicrobial activity was to do with the process of performing the LC/MS, the crude extract of *Streptomyces* FG1 was tested for acid sensitivity and to determine if it may become trapped on the column. This was performed as per Sections 2.24 and 2.25, respectively. Formic acid did not affect the ability for the extract to inhibit the indicator organisms, and biological activity was observed in the acetonitrile fraction of the C-18 Sep-Pack analysis, suggesting that neither formic acid nor the HPLC column were impacting the antimicrobial activity of the crude extract. This was confirmed when analytical LC/MS was performed to compare the processed samples with crude extract, revealing no significant difference in their chromatograms. Further study is needed to determine the cause of this loss of activity and to better understand which compound (or compounds) are responsible for the observed activity.

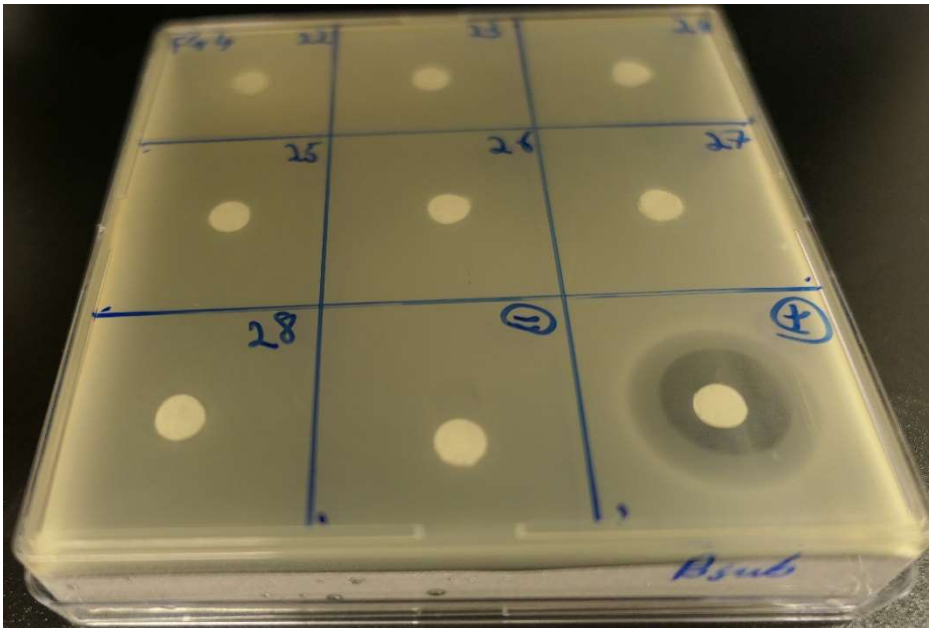


Figure 41 – Disk Diffusion Assay of Fractions 22-28 of *Streptomyces* FG4 Extract against *B. subtilis*. No fraction demonstrated the ability to inhibit the indicator strain.

4.3.2 Analysis of *Streptomyces* FG1 Extracts

As seen in Figure 42 and Figure 43, the two ethyl acetate extracts of *Streptomyces* FG1 appeared similar when analysed by LC/MS. Seventeen major peaks were identified, including what is suspected to be two pairs of structural isomers. The peaks with retention times of 7.124 and 7.251 minutes not only eluted at similar times but had similar UV spectra and MS fragmentation, with an [M+] of 540m/z. There were some differences in the negative ion MS spectra, most notably a lack of [M-] fragment in the later eluting peak. For the same reason, the peaks at 8.726 and 8.867 minutes were also believed to be isomers, although both with more similar negative ion MS results in this instance. Similar to the extracts of *Streptomyces* FG4, the *Streptomyces* FG1 samples were combined prior to fractionation. Figure 44 shows that fractionation of the *Streptomyces* FG1 extracts revealed that fractions 22-26 inhibited *B. subtilis* when bioassays were performed, as per Section 2.22. These fractions showed a similar pattern to those seen when testing *Streptomyces* FG4 – with each fraction showing comparatively weaker bioactivity than the crude extract. No activity was observed when the fractions from this extract were tested against *E. coli* or *C. albicans*, in line with the lack of activity observed in the crude extract. Potential explanations for this spread of antimicrobial activity have already been discussed in Section 4.3.1, where the fractionation of *Streptomyces* FG4 showed a similar pattern of results.

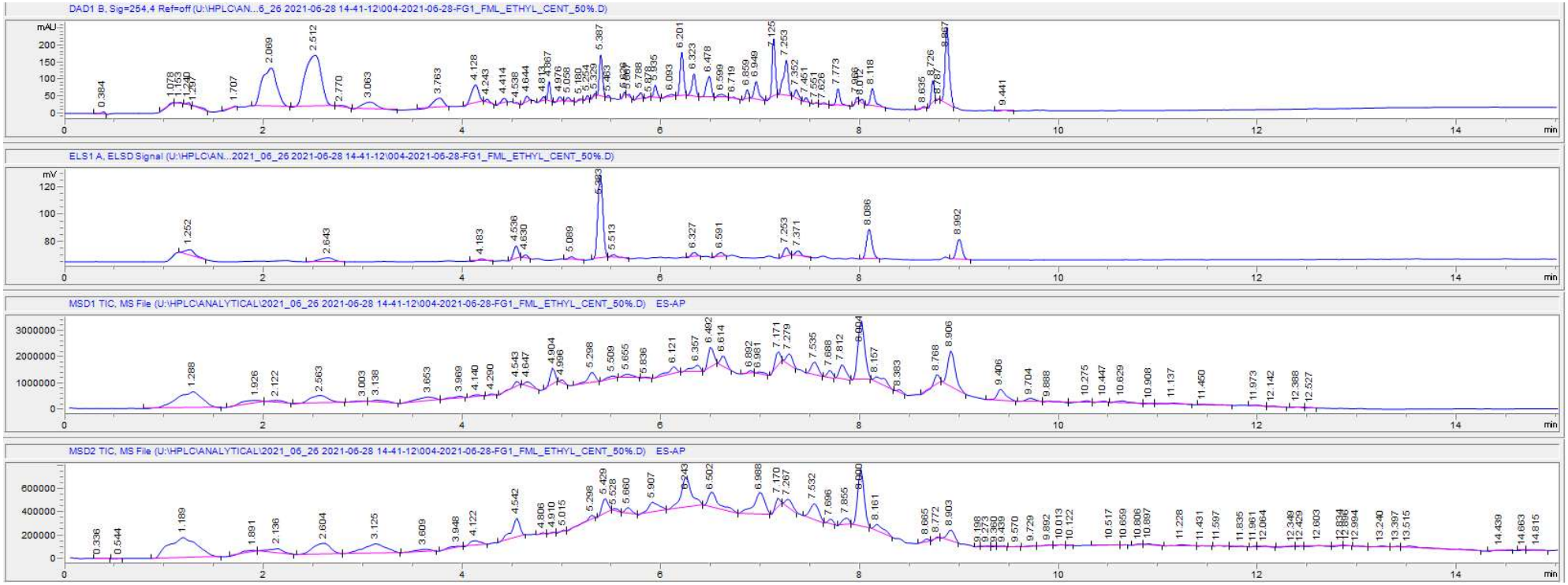


Figure 42 - Analytical LC/MS of the Ethyl Acetate Extract of *Streptomyces* FG1 Cultivated on FML, Sample A - with chromatograms from a (top) UV spectrometer at 254nm, (second from top) ELSD, (second from bottom) positive ionisation mass spectrometry and (bottom) negative ionisation mass spectrometry.

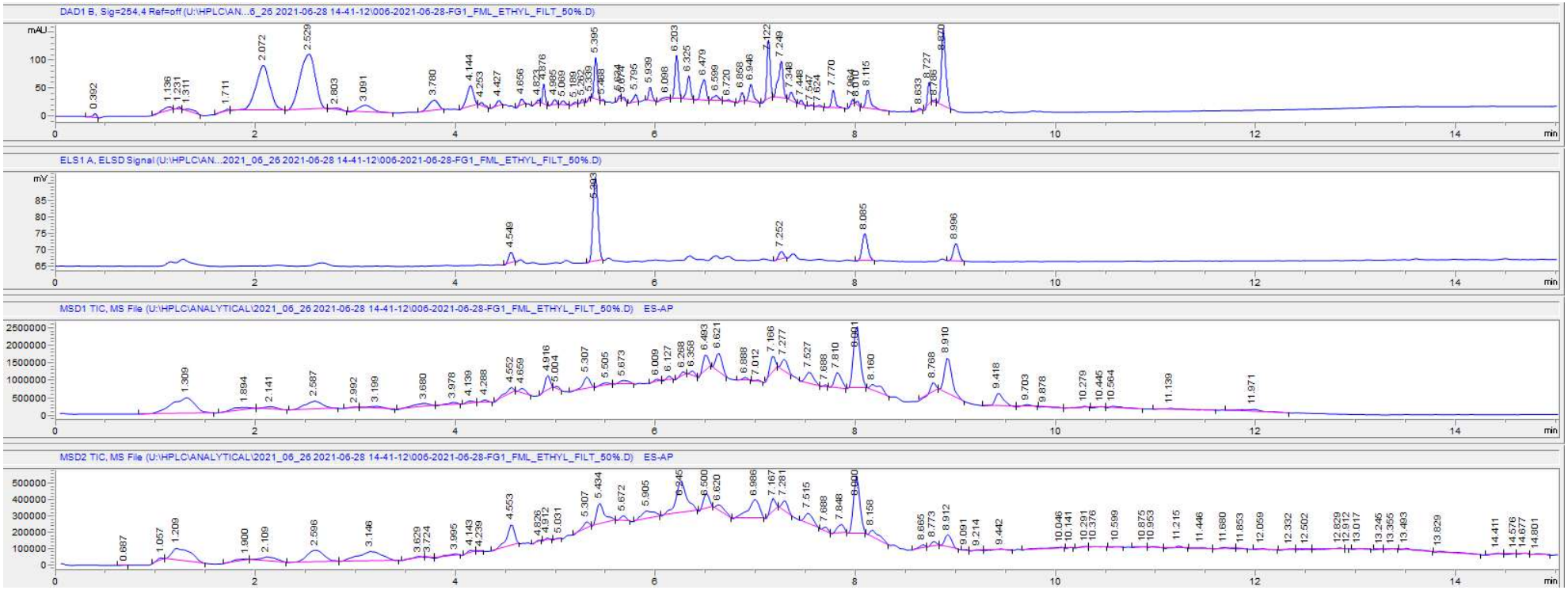


Figure 43 - Analytical LC/MS of the Ethyl Acetate Extract of *Streptomyces* FG1 Cultivated on FML, Sample B - with chromatograms from a (top) UV spectrometer at 254nm, (second from top) ELSD, (second from bottom) positive ionisation mass spectrometry and (bottom) negative ionisation mass spectrometry.

Table 41 - Summary of Major Peaks from the LC/MS analysis of both *Streptomyces* FG1 Extracts

Retention Time[1]	UV Absorption (nm)	ELSD (mV)[1]	MS+ (m/z)	MS- (m/z)
2.071	200, 250	0	281	279
2.520	350	70	209	207
3.771	190, 210, 250	0	299	301
4.126	210, 255	50	244	242
4.872	200, 275, 340, 410	70	265	-
5.391	210, 250, 315	130	267	463
5.937	210, 265, 365	0	244	-
6.202	210, 250	65	401	-
6.324	220, 260, 280	65	684	682
6.479	210, 260	65	718	-
6.947	215, 250	0	201	-
7.124	270, 330	70	540	538
7.251	270, 330	70	540	-
7.772	200, 270, 300, 30, 410	65	323	321, 773
8.117	230, 270, 340	75	868	870
8.726	275, 370	70	435	433
8.867	275, 370	70	435	433

1. The values reported here are the average of those observed in extracts A and B.

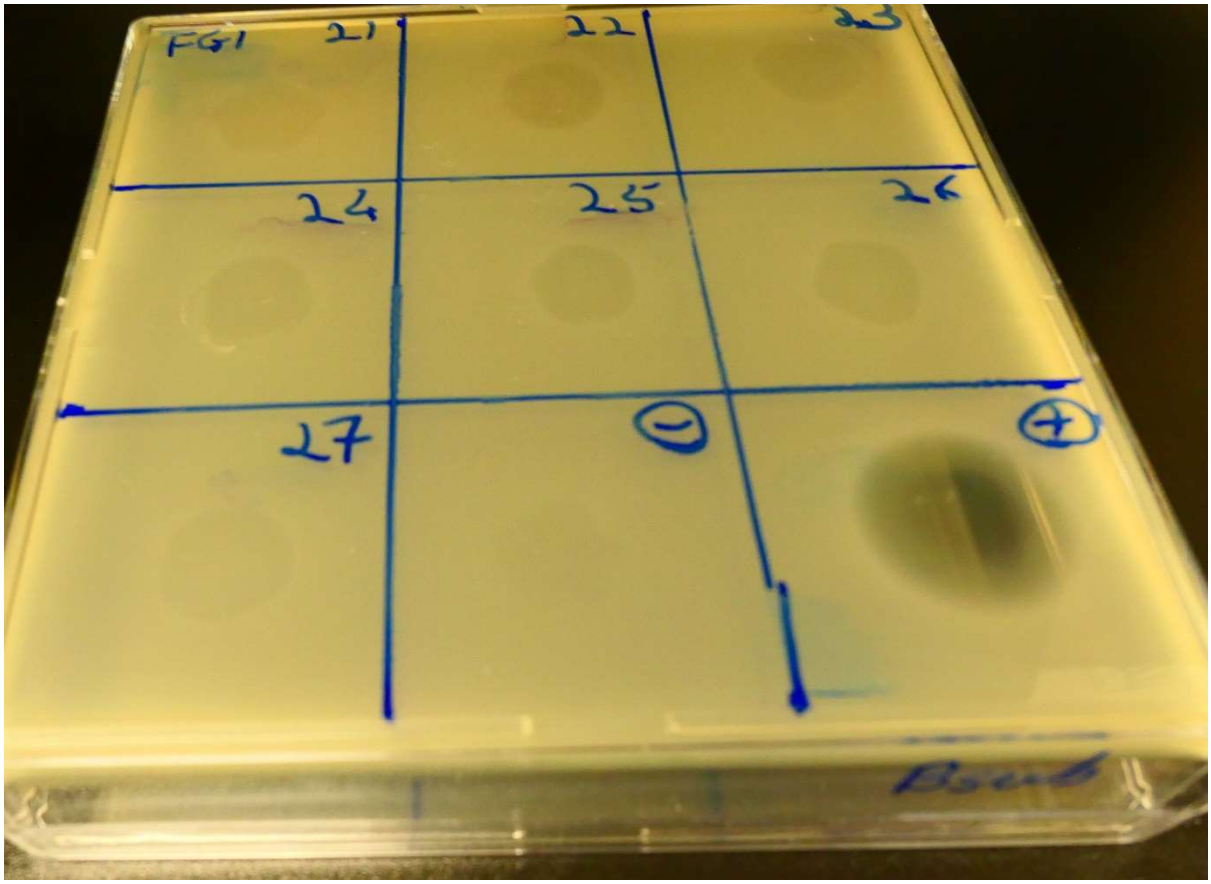


Figure 44 – Activity Assay of Fractions 21-27 of *Streptomyces* FG1 Extract against *B. subtilis*. Although fractions 21-27 showed some ability to inhibit the indicator strain, no fraction possessed a similar ability to that of the crude extract.

The effect of formic acid on the antimicrobial activity of the *Streptomyces* FG1 extract was tested, as was the potential for antimicrobial compounds becoming stuck on the HPLC column, as per Sections 2.24 and 2.25. Neither results could adequately explain the reduction of activity, with formic acid having no impact on the antimicrobial properties of the crude extract and antimicrobial activity being observed in the acetonitrile fraction of the C18 Sep-Pack chromatography. When samples before and after exposure to formic acid or fractionation through the C18 Sep-Pack were analysed using LC/MS, no significant difference was found, with the chromatograph in both cases being highly similar to those of the crude extracts. Unfortunately, lack of time prevented further investigation of this observation.

4.3.3 Analysis of *Amycolatopsis* UM15 Extracts

A single ethyl acetate extraction of *Amycolatopsis* UM15 after cultivation on FML was performed, which was then analysed by LC/MS. None of the peaks could be easily attributed to previously reported compounds, although LC/MS/MS would have to be performed to confirm this and to better analyse the compounds extracted. The ability of the *Amycolatopsis* UM15 extract to inhibit the growth of *B. subtilis* was weaker than the two *Streptomyces* extracts discussed above. Due to this and a lack of time, it was decided to focus on the *Streptomyces* extractions over the *Amycolatopsis* extract.

4.4 Discussion

In this chapter, chemical extractions of six different actinomycetes were conducted to purify the antimicrobial compounds they produce. For *Streptomyces* KY2, B2 and A7, biologically active extracted were only produced when the actinomycetes were challenged by the indicator strain they were able to inhibit. Biologically active extractions from *Streptomyces* FG4, FG1 and *Amycolatopsis* UM15 were produced from axenic cultures using ethyl acetate. Although ethyl acetate and methanol extracted antimicrobial activities from all the tested strains, ethyl acetate was able to extract compounds responsible for antimicrobial activity from a wider range of strains and conditions, often with higher yields. Additionally, only ethyl acetate extractions inhibited the growth of *C. albicans.* No extractions performed by freezing then thawing the media or using water as a solvent extracted antimicrobial activity. This potentially could be due to the extraction process degrading the produced antimicrobial and, given the ability for both methanol and water to perform nucleophilic substitutions, this may explain why ethyl acetate extracted biological activity from a wider variety of conditions. Alternatively, the observed microbial inhibition could have been caused enzymatically or due to siderophore iron sequestering, which would not have been extracted by the methods used here. This would explain why activity from many strains was difficult to extract on some of the media used if the antimicrobial activity on some media was caused this way and on others caused by an antimicrobial compound. In order to obtain biologically active extracts, several stages of optimising the extraction method were required, including

replacing filter paper with centrifugation to remove particulate debris. This implies that the filter paper absorbed the antimicrobial compounds being produced in sufficient quantities to reduce the compound's concentration to below the MIC. Additionally, placing the extraction mix on a reciprocating shaker increased yields, presumably from increased diffusion rates out of the media and into the solvent. Extraction flasks were also protected from UV light by wrapping the containers in multiple layers of blue roll. Although it is not clear if this improved yields or antimicrobial abilities of the extracts, given UV light's ability to degrade many compounds, this is probably a prudent measure when chemical extractions are proving challenging.. It should be noted that the concentration of extractions between conditions was not maintained, so there is not possible to compare the activity of extractions from different strains with each other. It would have been preferable to standardise this to allow comparison, and potentially assist with the optimisation of the HPLC analysis. Within a fractionation, however, concentration was maintained between fractions and the initial crude extract – which does allow for comparisons to be drawn.

Ethyl acetate extracts from *Streptomyces* FG4, FG1 and *Amycolatopsis* UM15 that demonstrated antimicrobial activity were analysed using LC/MS, with no constituent chemicals detected being linked to known antimicrobials – although LC/MS/MS was not able to be performed on the *Streptomyces* FG1 and *Amycolatopsis* UM15 extracts to conclusively rule-out the potential that a previously-reported antimicrobial had been rediscovered. LC/MS analysis of duplicate extractions of *Streptomyces* FG1 and FG4 provided highly similar chromatograms, suggesting that similar compounds are produced consistently by these strains, allowing future work to be more easily performed. For both *Streptomyces* extracts, activity-guided fractionation was attempted to purify the antimicrobial compound. In both cases, multiple fractions possessed antimicrobial activity. To determine if this was due to the antimicrobial compound becoming stuck on the HPLC column, a C-18 sep-pack was used, although in both cases, antimicrobial activity was present in the acetonitrile fraction, suggesting that this is not the case. Multiple compounds may combine to produce the observed antimicrobial effect, rather than a single molecule, and these elute with

different retention times. These compounds may be encoded by a single smBGC, with some acting as intermediates but still processing antimicrobial properties, similarly to the friulimicin A/B/C/D encoding BGC in *Actinoplanes friuliensis* (Schneider *et al.*, 2009). If this is the case, normal-phase HPLC or an alternative chromatography method, such as flash chromatography, may prove better able to separate the compounds.

Although attempts were made to acquire LC/MS/MS data of the extracts, these ultimately proved unsuccessful. This was due to a variety of reasons. During the first attempts to obtain LC/MS/MS data, a Pourous C18 30x4.6mm column was incorrectly fitted to the instrument, and the error was not discovered for several weeks. This column is intended for preparatory HPLC, thus providing insufficient resolution for accurate LC/MS/MS analysis. Instead, a Kinatex 1.7µm column was intended to be used. Further attempts to perform the LC/MS/MS were hindered by machine faults and breakages, taking the instrument offline for several weeks. Given more time, a full-LC/MS/MS analysis would be performed on all extracts to better understand the chemical composition of the extracts and how they are related to previously reported compounds. This would also aid with ruling out previously-reported chemicals when attempting to isolate the antimicrobials and, if more extracts were performed, allow for statistical analysis of detected compounds to determine the likelihood they are related to previously reported compounds. Efforts to purify the antimicrobial compound should also continue; a flash chromatograph, for example, could be used to perform an initial fractionation, generating a few larger fractions of extract for analysis. This would allow all the antimicrobial fractions observed during HPLC-based fractionation to be analysed simultaneously whilst still making steps towards purification. If multiple compounds are responsible for the antimicrobial activity, this may also result in the antimicrobial activity being easier to observe due to the increased potency. Alternatively, higher concentrations of the compounds could be generated by producing increased quantities of extract and combining them prior to fractionation. This would make antimicrobial activity easier to observe, particularly if some fractions contain more potent antimicrobials than others. Additionally, given that only one extraction was successfully performed here, more extractions from

Amycolatopsis UM15 should be conducted to ensure consistency of the extract and allow for statistical LC/MS/MS analysis.

Once purification has been completed, the compound's mechanism of action could be identified initially by utilising *Bacillus* reporter strains such as *B. subtilis* PL39 gyrA::pMUTIN4 ermC gyrA'-lacZ PspacgyrA+, a DNA gyrase inhibition reporter and *B. subtilis* ypuA::pMUTIN4 ermC ypuA'-lacZ, a cell wall damage reporter, among others. This would indicate the mechanism of action for the antimicrobials encoded by the actinomycetes studied here and inform further work to determine antimicrobial characteristics, such as the MIC and structure. This would then help with the identification of the smBGC encoding the antimicrobial by cross-referring this data with the antiSMASH analysis performed in Section 3.3.

5 Genetic Modification of Two *Streptomyces* Strains Using CRISPR/Cas9

In Chapter 3, 18 actinomycetes associated with fungus-growing ants were tested for their biological activities against *B. subtilis, E. coli* and *C. albicans* across 33 different growth media. This was then compared to the smBGCs predicted to be encoded in the genomes when they were analysed by antiSMASH to determine which strains had an smBGC present with high homology to a previously reported smBGC encoding an antimicrobial. For some strains, no smBGCs had high homology to known antimicrobial encoding smBGCs; for others, they were predicted to contain at least one smBGC with high homology to a previously reported antimicrobial encoding smBGC. In this chapter, those strains with smBGCs that showed high similarity to smBGCs known to encode for antimicrobials were assessed for their suitability as candidates for genetic modification using CRISPR/Cas9 with the objective of knocking out the smBGCs suspected to encode antimicrobials. This was performed first to determine if that smBGC was responsible for the antimicrobial activity observed and secondly to determine which compound the BGC was encoding. If antimicrobial activity ceased after the smBGC was deleted, this would indicate that it is likely that the compound it is encoding is responsible for the observed activity. This would allow for comparative metabolomics to be performed to determine the compound responsible for the antimicrobial activity, potentially allowing for purification – the overall aim of this chapter.

Genetic modification of *Streptomyces* strains can prove difficult compared to other bacterial species due to only being susceptible to modification after sporulation and possessing a relatively large genome with an estimated 5-40% of that genome not confined to plasmids. Further, *Streptomyces* shows high phenotypic and genotypic variability, making the selection and confirmation of mutants more challenging. (Parish, 1986; Reeve, 1986; Kieser *et al.*, 2000) Historically, gene disruption in *Streptomyces* relied upon either single crossover integration of a suicide plasmid or larger-scale double-crossover integrations used for deletions of genomic information. The issue with using suicide vectors in *Streptomyces* is that restoration of the wild-type is common if selective pressure is removed. To counter this, recombinase target sites in the surrounding DNA can be over-expressed to improve the stability of the mutation, although this approach can lead to scarring of the genome. Additionally, the reusability of suicide vectors is limited by the number of selectable markers in the target gene.

On the other hand, double-crossover integration is a time-consuming and labour-intensive process where one must first integrate the disruption vector at the target using a selective marker. Then a non-selective medium must be used to all for the loss of the disruption vector, the colonies of which must be further screened to see if that loss has resulted in a reversal to wild-type or the desired modification (Kieser *et al.*, 2000; Cobb, Wang and Zhao, 2015). For rare actinomycetes, there are even fewer tools available for genetic modification and these are often challenging to conjugate plasmids into, making genetic modification even more difficult than *Streptomyces*. To improve these methods, a specialised CRISPR/Cas9 plasmid – the pCRISPomyces-2 plasmid – was developed from the CRISPR/Cas9 system of *Streptococcus pyogenes* to allow for targeted editing of *Streptomyces* genomes with lower labour time than previous methods. The second-generation pCRISPomyces-2 plasmid includes a single-guide RNA expression cassette and a *Cas9,* unlike the pCRISPomyces-1, which included tracrRNA and a full CRISPR array expression cassette but required a separate *Cas9* containing plasmid (Cobb, Wang and Zhao, 2015). This reduces the number of elements that need to be conjugated into the *Streptomyces* strain being modified.

If a BGC encoding an antimicrobial is successfully knocked-out using CRISPR/Cas9, it can reveal some important information. Firstly, it would identify the BGC encoding the antimicrobial, allowing for further study of said BGC to better understand the structure of the chemical produced and, in time, feeding this information into future analysis of actinomycete genomes. Secondly, preventing the biosynthesis of the antimicrobial allows for a comparative metabolomics study to be performed – comparing the extracts produced by the wild-type and conjugant strains to identify the antimicrobial compound. Finally, much is still to be learnt about the genetic modification of non-model *Streptomyces* species; by genetically modifying these ant-associated strains, it is hoped that alternative methodologies can be determined to better our understanding of modifying these organisms.

Dr Rebecca Devine kindly generated the strains discussed in this chapter. All other analysis was conducted by the author.

5.1 Selecting CRISPR/Cas9 Targets

In order to determine which strains were likely to be genetically tractable, all 18 actinomycetes were challenged by common selection markers used during the CRISPR/Cas9

protocol – apramycin and hygromycin at 0.25, 0.5, 1 and 2x concentrations to those described in Section 2.1. As shown in Table 42, 10 strains were susceptible to both apramycin and hygromycin, even at one-quarter of their normal concentrations. As can be seen in Figure 45, both *Amycolatopsis* strains demonstrated some resistance to hygromycin, with *Amycolatopsis* UM15 resistant to twice the normal concentration. *Streptomyces* A7 was resistant to apramycin at all the concentrations tested here. This means that none of *Streptomyces* A7, *Amycolatopsis* UM15 and FG22 were likely to be highly genetically tractable or require newly designed plasmids with different antibiotic selective markers encoded.

Table 42 - Antibiotic Susceptibility of the 18 Ant-Associated Actinomycetes. "S" refers to a strain that was susceptible to the antibiotic, "P" is partial resistance – some colonies were able to survive, but not all, and "R" are strains that showed complete resistance to the antimicrobial at that concentration. Strains that were able to resist either antibiotic at any concentration were not selected for modification using the pCRISPomyces-2 system. *Streptomyces* A7 was able to resistant apramycin even at twice the concentration usually utilised. Similarly, *Amycolatopsis* UM15 was able to resist hygromycin at twice the usual concentration.

Actinomycete	Apramycin Concentration (µg/mL)				Hygromycin Concentration (µg/mL)			
	12.5	25	50	100	12.5	25	50	100
Pseudonocardia UM4	S	S	S	S	S	S	S	S
Pseudonocardia UM14	S	S	S	S	S	S	S	S
Pseudonocardia UM9	S	S	S	S	S	S	S	S
Pseudonocardia P1	S	S	S	S	R	P	S	S
Streptomyces KY2	S	S	S	S	S	S	S	S
Streptomyces B2	R	P	S	S	R	S	S	S
Streptomyces FG4	S	S	S	S	S	S	S	S
Streptomyces FG7	S	S	S	S	R	P	S	S
Streptomyces A7	R	R	R	R	R	R	S	S
Streptomyces KY4	S	S	S	S	S	S	S	S
Streptomyces FG1	S	S	S	S	S	S	S	S
Streptomyces KY1	S	S	S	S	R	S	S	S
Amycolatopsis UM15	P	P	S	S	R	R	R	R
Amycolatopsis FG22	P	S	S	S	R	P	P	S
Agrococcus A6	S	S	S	S	S	S	S	S
Tsukamurella FG11	S	S	S	S	S	S	S	S
Tsukamurella E7	S	S	S	S	S	S	S	S
Jiangella S1	S	S	S	S	P	P	S	S

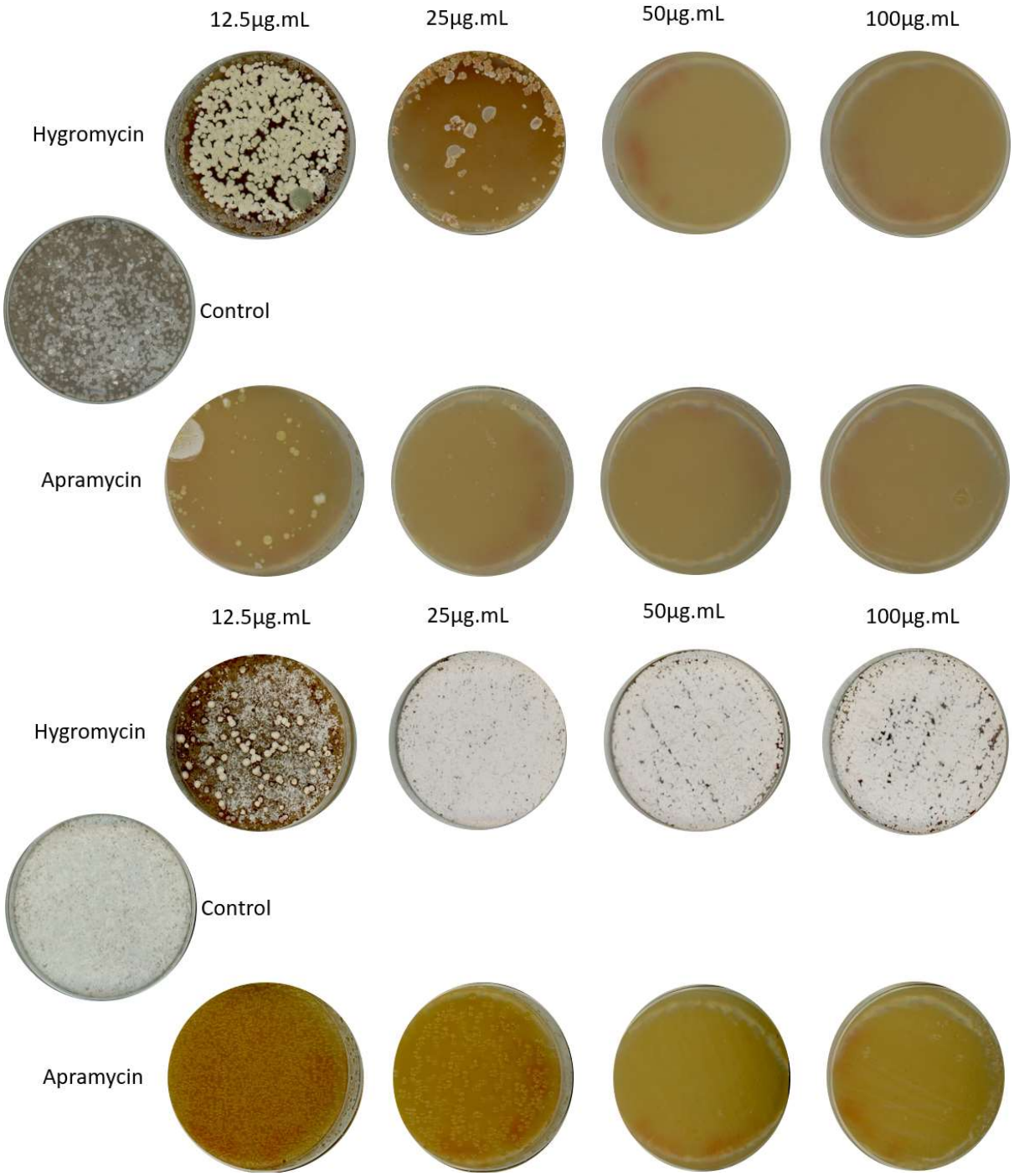


Figure 45 - Antimicrobial Susceptibility of *Amycolatopsis* FG22 (Above) and UM15 (Below) to hygromycin and apramycin. Based on these observations, *Amycolatopsis* FG22 was deemed to be resistant to hygromycin at 12.5µg/mL, partly resistant at 25µg/mL and susceptible to 50µg/mL and higher concentrations, whilst being partially resistant to 12.5µg/mL apramycin and susceptible to concentrations higher than 25µg/mL. *Amycolatopsis* UM15 was deemed resistant to hygromycin at all concentrations tested here whilst being resistant to apramycin concentrations up to 25µg/mL and susceptible to 50µg/mL and higher concentrations.

As detailed in Section 3, the time to sporulation for the different strains cultivated on solid SFM had also been determined. A shorter time for sporulation would allow for easier genetic manipulation of the strains, given the need for sporulation before genetic modification can

be performed. This discouraged the rare actinomycetes *Agrococcus* A6, *Tsukamurella* A6 and FG11 and *Jiangella* S1 from being selected due to their long time to sporulation, of between 17 and 20 days. The strains that sporulated most rapidly after inoculation on SFM were *Streptomyces* KY2 and FG4, both taking seven days to sporulate, with *Streptomyces* B2, FG7 and FG1 all requiring eight. None of these were resistant to hygromycin or apramycin at selective concentrations, although *Streptomyces* B2 showed some ability to resist low concentrations of apramycin and *Streptomyces* FG7 of hygromycin.

The antiSMASH predictions of *Streptomyces* KY2, FG4 and FG1, performed in Section 3, were then compared to determine which strains had the highest potential for smBGCs encoding suspected antimicrobial that could be targeted. For *Streptomyces* FG1, multiple smBGCs were identified as potential sources of antimicrobial activity; Region 5, with 91% homology to the valinomycin encoding smBGC, Region 7, with 100% homology to the SGR-PTM encoding smBGC, Region 8, with 100% similarity to the bafilomycin B1 encoding smBGC and, Region 18, with 100% homology to the keywimysin encoding smBGC. With four smBGCs with the potential to encode compounds with antimicrobial activity, it is possible to target a region that was predicted to encode an antimicrobial that was not being expressed, and thus knocking out using CRISPR/Cas9 would have had a minimal effect on the observed phenotype as the expressed antimicrobial would be unaffected. *Streptomyces* KY2 was predicted to possess only two such smBGCs – one antibacterial in Region 16, which had an 87% homology to the puromycin encoding smBGC and one antifungal in Region 29, which had a 76% similarity to the candicidin encoding smBGC. Only the antibacterial or antifungal activity should be affected by targeting these separately. Meanwhile, *Streptomyces* FG4 was predicted to contain just two smBGCs with the potential to encode for antimicrobials; Region 1.10, with 100% homology to albaflavenone and Region 18, with 61% homology to the kanamycin encoding smBGC. That *Streptomyces* FG1 possessed a relatively high number of smBGCs potentially encoding antimicrobials meant that it was determined less likely that a successful knockout of any one region would change the antimicrobial phenotype observed. Thus, *Streptomyces* FG4 and KY2 were selected for genetic modification attempts.

Region 18 of *Streptomyces* FG4, predicted to have a 61% similarity to the kanamycin BGC in *S. kanamyceticus* 12-6 was selected to be targeted for CRISPR/Cas9 modification. This region was selected because *Streptomyces* FG4 demonstrated the ability to inhibit *E. coli* when

cultivated on Min, SM6, SM12, minNAG and GYM + 10µg/mL streptomycin, and the antiSMASH analysis showed only Region 18 had any similarity to an antibacterial agent known to inhibit Gram-negative bacteria, this is discussed in section 3.3.7. Region 18 was predicted to be 41,700bp in length and contain 34 genes. Of these, however, only eight genes, totalling 9149bp, were deemed to have high homology to those present in the kanamycin BGC. As shown in Figure 46, antiSMASH predicted that a T1PKS gene – designated *ctg1_4838* - formed the core biosynthetic gene, which had a 98% similarity to a malonyl-CoA acyltransferase protein encoded in *S. bauhiniae* and *E. coli* when aligned in BlastP, as seen in Table 43. This is despite kanamycin being an aminoglycoside, with the encoding BGC not containing a PKS cluster.

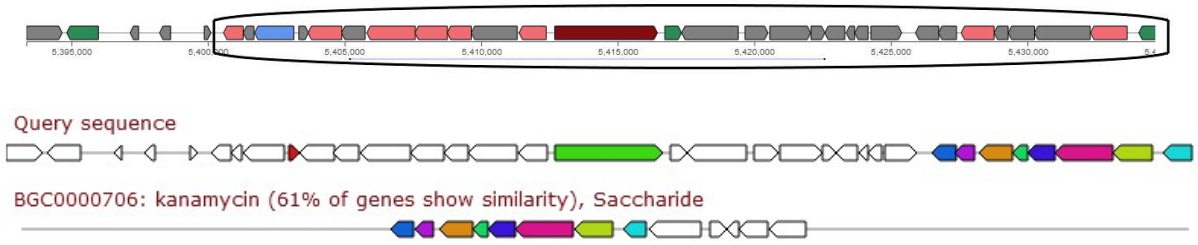


Figure 46 - Region 18 of *Streptomyces* FG4 Predicted by antiSMASH (top) and Compared to the Kanamycin smBGC (bottom). In the top image, dark red genes are those predicted by antiSMASH to be a core biosynthetic gene for the region, pale red additional biosynthetic genes, blue transport-related genes and green regulatory genes, whilst grey genes are those that do not fit into these categories. The black ring encircles the hypothecated smBGC encoded in Region 18.

Table 43 - BlastP Analysis of *ctg1_4838* showing the high homology with multiple malonyl-CoA acyltransferases across *Streptomyces* species. This suggested that, despite Region 18 having high homology to the aminoglycide kanamycin, there were some genes present that more closely resembled PKS biosynthetic machinery.

Description	Scientific Name	Query Cover (%)	E value	Identity (%)	Accession
acyltransferase domain-containing protein	*S. bauhiniae*	98	0	93.23	WP_164188109.1
acyltransferase domain-containing protein	*S. bauhiniae*	98	0	93.63	WP_135786154.1
acyltransferase domain-containing protein	*S. griseoluteus*	98	0	93.23	WP_135794604.1
type I polyketide synthase	*Streptomyces sp.* NRRL F-2799	98	0	92.58	WP_051720403.1
type I polyketide synthase	*S. recifensis*	98	0	92.82	WP_086695547.1

Additionally, the bulk of the genes with high homology to those in the kanamycin encoding smBGC are separated from *ctg1_4838* by 9452bp, as seen in Figure 46, being found at the edge of Region 18. This may suggest that *ctg1_4838* is not a core biosynthetic gene. Of the genes that are present, all have high homology to the genes in the kanamycin encoding smBGC, presented in Table 44, the lowest being *DJ545_24920*, with a 96.92% homology to a TetR family regulator encoding gene. Although the nearest relative to the genes analysed here with BlastP do not originate from the kanamycin encoding smBGC in *S. kanamyceticus,* they serve the same function as equivalently located genes in the kanamycin smBGC. None of the genes in the kanamycin smBGC but missing from Region 18 are present in the *Streptomyces* FG4 genome within 1000bp of Region 18, suggesting antiSMASH did not dissect a complete kanamycin encoding smBGC. Five genes in the kanamycin smBGC were absent in Region 18 of *Streptomyces* FG4; *BAE95600.1*, encoding a glycosyltransferase, *BAE95601.1*, *BAE95602.1* and *BAE95603.1*, all encoding unknown proteins and *BAE95604.1*, encoding a putative dehydrogenase. The lack of glycosyltransferase may indicate that kanamycin is not being produced.

Table 44 - Top BlastP Result for Each Gene antiSMASH Predicted to be Part of a Kanamycin Encoding smBGC. All genes in *ctg1_4838* showed a high homology to genes in other actinomycetes that encode proteins with similar functions to those in the kanamycin encoding smBGC.

antiSMASH Designation	Description	Scientific Name	Query Cover (%)	E value	Identity (%)	Accession
DJ545_24920	TetR family transcriptional regulator	*S. bauhiniae*	100%	0	96.92	TGN76449.1
DJ545_24915	crotonyl-CoA carboxylase/reductase	*S. sp. SID2999*	100%	0	100.00	MYZ06271.1
DJ545_24910	protein meaA	*S. sp. SID2999*	100%	0	99.85	MYZ06272.1
DJ545_24905	CoA ester lyase	*S. sp. NRRL F-2799*	100%	0	99.38	WP_030809538.1
DJ545_24900	MaoC family dehydratase	*Streptomyces. spp.*	100%	3.00^{-123}	99.41	WP_030809541.1
DJ545_24895	acyl-CoA dehydrogenase family protein	*S. bauhiniae*	100%	0	99.75	WP_164188118.1
DJ545_24890	phosphatidylserine decarboxylase	*Streptomyces. spp*	100%	1.00^{-130}	100.00	WP_031183665.1
DJ545_24885	CDP-diacylglycerol--serine O-phosphatidyltransferase	*S. griseosporeus*	100%	1.00^{-179}	97.18	GHF45292.1

It was theorised that Region 18 might encode an smBGC combining *ctg1_4838,* the genes found in the kanamycin smBGC, the nine genes between these regions and then as far as *DJ545_24780*, predicted to encode a short chain dehydrogenase. Seven of the nine genes between *ctg1_4838* and the genes present in the kanamycin encoding smBGC are known to encode biosynthetic proteins, summarised in Table 45. One – *DJ545_24870* - has an unknown purpose, and one – *DJ545_24840* – encodes a TetR family regulator. Seven of these genes, when analysed by BlastP, found their most highly similar protein to originate from *S. bauhiniae sp.*, isolated from the bark of the *Bauhinia variegate* tree in Thailand (Kanchanasin *et al.*, 2020). Those genes between *ctg1_4838* and *DJ545_24780* are summarised in Table 46, showing only one – *DJ545_24810* – not having a function predicted. *DJ545_24820* was predicted to encode for a DHA2 family multi-drug efflux pump, which may act as a transporter protein to export the product of the hypothecated smBGC, whilst the other nine genes are predicted to encode biosynthetic enzymes. If all of these genes are part of the smBGC, it would total 34,483bp and contain 29 genes.

Table 45 - Top BlastP Result for Each Gene Between *ctg1_4838* and the Gene with High Homology to Those Found in the Kanamycin smBGC. These genes may connect the genes with high homology with the kanamycin smBGC and *ctg_4838* into one, larger, smBGC encoding a novel antimicrobial.

antiSMASH Designation	Description	Scientific Name	Query Cover (%)	E value	Identity (%)	Accession
DJ545_24840	TetR/AcrR family transcriptional regulator	*S. bauhiniae*	100	5.00^{-147}	99.51	TGN76861.1
DJ545_24845	hypothetical protein	*S. bauhiniae*	100	0	96.08	WP_135786153.1
DJ545_24850	methylenetetrahydrofolate reductase	*S. bauhiniae*	100	1.00^{-167}	95.70	TGN76860.1
DJ545_24855	helix-turn-helix domain-containing protein	*S. bauhiniae*	100	0	96.59	WP_135786151.1
DJ545_24860	methylated-DNA--[protein]-cysteine S-methyltransferase	*S. sp.* SID2999	100	1.00^{-103}	97.63	MYZ09033.1
DJ545_24865	NAD-dependent deacetylase	*S. bauhiniae*	100	2.00^{-154}	94.24	WP_164188113.1
DJ545_24870	hypothetical protein	*S. seoulensis*	53	6.00^{-25}	90.91	WP_158713910.1
DJ545_24875	NUDIX hydrolase	*S. bauhiniae*	100	3.00^{-107}	97.56	WP_135786147.1
DJ545_24880	glycerate kinase	*S. bauhiniae*	98	4.00^{-169}	94.59	WP_164188117.1

Table 46 - Top BlastP Result for Each Gene Between *ctg1_4838* and *DJ545_24780.* These genes may be part of a larger smBGC, alongside those detail in Table 45, to encode a novel antimicrobial.

antiSMASH Designation	Description	Scientific Name	Query Cover (%)	E value	Identity (%)	Accession
DJ545_24780	aldo/keto reductase	*S. griseoluteus*	100	0	98.18	WP_135794605.1
DJ545_24785	GH3 auxin-responsive promoter family protein	*S. bauhiniae*	100	0	98.40	WP_135786156.1
DJ545_24790	alpha/beta fold hydrolase	*S. bauhiniae*	100	0	98.29	WP_135786157.1
DJ545_24795	mandelate racemase	*S. bauhiniae*	97	0	95.05	WP_164188105.1
DJ545_24800	thiamine pyrophosphate-binding protein	*S. griseoluteus*	100	0	97.97	TGN74141.1
DJ545_24805	FAH family protein	*S. bauhiniae*	100	0	97.55	WP_164188103.1
DJ545_24810	hypothetical protein	*S. griseoluteus*	100	0	91.26	GHF08404.1
DJ545_24815	helix-turn-helix domain-containing protein	*S. bauhiniae*	100	6.00^{-68}	97.44	WP_164188101.1
DJ545_24820	DHA2 family efflux MFS transporter permease	*S. sp.* SID1328	100	0	98.12	MYV40783.1
DJ545_24825	5-carboxymethyl-2-hydroxymuconate Delta-isomerase	*S. sp.* NRRL F-2799	100	1.00^{-76}	97.69	WP_051720405.1
DJ545_24830	SDR family oxidoreductase	*S. bauhiniae*	99	6.00^{-150}	97.06	WP_135786165.1

It was speculated that Region 18 may encode for an antimicrobial structure, potentially with a similar pharmacophore to that of kanamycin, which possessed the ability to inhibit kanamycin-resistant *E. coli*, as discussed in Section 3. Thus, it was hypothesised that deletion of the *ctg1_4838* would result in the loss of antimicrobial activity by preventing the biosynthesis of the encoded compound. In order to minimise disruption to global regulation, it was decided to target *ctg1_4838* alone, totalling 3,758bp, rather than remove the larger BGC. This also meant that finding defined edges of the BGC was not required. Given the relative significance of this gene in the BGC, it was deemed likely that its removal would disrupt the produced metabolite significantly enough to remove the product's antimicrobial activity or prevent its biosynthesis entirely.

The other region targeted for knockout using CRISPR/Cas9 was Region 16 of *Streptomyces* KY2, visualised in Figure 47. The antiSMASH analysis, presented in Section 3.3.5, predicted Region 16 to have an 87% similarity to the smBGC encoding puromycin, which one would expect to easily extract when using water as a solvent in Section 4; however, this was not observed with water extractions of *Streptomyces* KY2 failing to demonstrate antibacterial activity. This increased the interest in *Streptomyces* KY2 as there was a suspicion that an alternative antimicrobial was being produced that would be more challenging to extract. As shown in

Table **47**, when the genes predicted by antiSMASH to be part of the puromycin smBGC were analysed by BlastP, the top result was not from the *S. alboniger* puromycin smBGC. Instead, other proteins encoded by *Streptomyces* species with similar roles were found to have a higher homology. Table 48 contains the BlastP results when the genes in Region 16 of *Streptomyces* KY2 were compared to the equivalent gene within the puromycin smBGC in *S. alboniger.* It was decided that the core biosynthetic gene, assigned *ctgl_2919* by antiSMASH and with high homology to *pur6*, would be targeted for knock-out using CRISPR/Cas9. *pur6* encodes a tyrosinyl-aminonucleoside synthetase, involved in the addition of tyrosine to the intermediate 3'-amino-3'-dA, and is essential for the production of puromycin (Ángel Rubio *et al.*, 2004). Similarly to the knock-out performed in *Streptomyces* FG4, *pur6* was targeted to minimise the disruption to global regulation but was still likely to prevent biosynthesis of the compound encoded by the region.

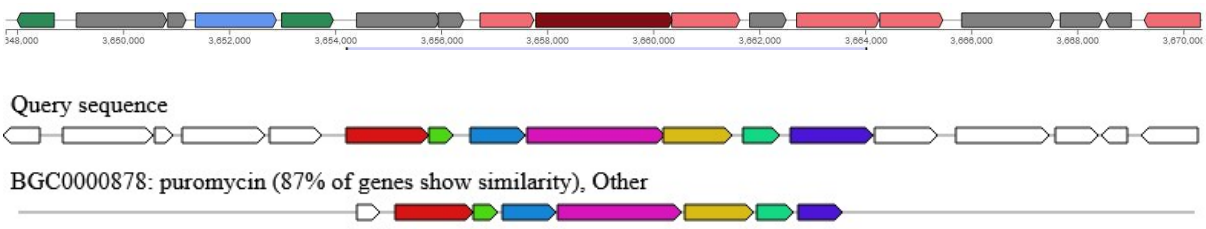


Figure 47 - Region 16 of *Streptomyces* KY2 Predicted by antiSMASH (above) and a Comparison of Region 16 with the Puromycin-Encoding BGC in *S. alboniger* (below). In the top image, dark red genes are those predicted by antiSMASH to be a core biosynthetic gene for the region, pale red additional biosynthetic genes, blue transport-related genes and green regulatory genes, whilst grey genes are those that do not fit into these categories. The bottom image shows that Region 16 lacks *orfA* that is present in the puromycin smBGC, which, as discussed in Section 3.3.5, is unlikely to be involved in the biosynthesis of puromycin.

Table 47 – Top BlastP Result of the Genes Predicted by antiSMASH to have High Homology to the Puromycin smBGC. All genes in *ctg1_2919* showed a high homology to genes in other actinomycetes that encode proteins with similar functions to those in the puromycin encoding smBGC.

antiSMASH Designation	Description	Scientific Name	Query Cover (%)	E value	Identity (%)	Accession
ctg1_2916	M28 family peptidase	*S. cacaoi*	92	0	99.17	WP_141275606.1
ctg1_2917	NUDIX domain-containing protein	*S. sp*. NRRL S-1868	100	6.00^{-108}	100.00	WP_030888599.1
ctg1_2918	Gfo/Idh/MocA family oxidoreductase	*Streptomyces sp*.	100	0	100.00	WP_030888602.1
ctg1_2919	hypothetical protein	*S. sp*. NRRL S-1868	100	0	97.47	WP_030888603.1
ctg1_2920	DegT/DnrJ/EryC1/StrS family aminotransferase	*Streptomyces sp.*	100	0	99.77	WP_030885426.1
ctg1_2921	methyltransferase	*Streptomyces sp.*	100	4.00^{-166}	100.00	WP_030885428.1
ctg1_2922	histidinol-phosphatase	*S. sp. NHF165*	100	0	99.42	QHF97863.1

Table 48 - BlastP of the Genes in *Streptomyces* KY2 Region 16 and Those in the Puromycin smBGC in *S. alboniger*. These revealed that the genes in Region 16 had high homology to those in the puromycin smBGC, and are thus likely to encode proteins with similar functions.

antiSMASH Designation	Equivalent *S. alboniger* gene	Description	Query Cover (%)	E value	Identity (%)	Accession
ctg1_2916	*napH*	M28 family metallopeptidase	83	4^{-171}	70.11	WP_167532748.1
ctg1_2917	*pur7*	NUDIX domain-containing protein	93	2.00^{-62}	68.71	WP_055528328.1
ctg1_2918	*pur10*	Gfo/Idh/MocA family oxidoreductase	99	4^{-157}	72.11	WP_246201669.1
ctg1_2919	*pur6*	hypothetical protein	87	0	76.11	WP_055528327.1
ctg1_2920	*pur4*	DegT/DnrJ/EryC1/StrS family aminotransferase	99	0	79.91	WP_055528325.1
ctg1_2921	*pur5*	methyltransferase domain-containing protein	98	7.00^{-129}	85.40	QEV22614.1
ctg1_2922	*pur3*	histidinol-phosphatase	45	2^{-106}	74.89	WP_055528323.1

Region 16 was predicted to be 22,576bp in length and contain 17 genes, with 7 of these, totalling 9,856bp, having high homology to the puromycin BGC. By targeting *ctgl_2919*,

2,577bp were planned to be knocked out from the genome to minimise disruption to global regulation.

For both strains, the design and assembly of the pCRISPomyces-2 plasmids were performed by the same method. Firstly, approximately 20 nucleotide-long gRNA was designed on the anti-coding DNA strand with a T_m of more than 60°C, and the genome was interrogated with all four potential PAM sequence NGG combinations to ensure there was no duplication of the target site. Once annealed, these were assembled into the BbsI cut site in the pCRISPomyces-2 vector using golden gate assembly. Additionally, a 2kb homology repair template was designed by taking the anti-coding strand of the target gene and 1kb on either side, then deleting and replacing the targeted gene with a linker sequence, maintaining the start and stop codons to reduce the risk of frameshift mutations. From this sequence, primers were designed as described in Section 2.15 to enable the construction of the repair template and its assembly via Gibson assembly and XbaI. The final vector was cloned into the non-methylating *E. coli* ET12567/pUZ8002 and confirmed by PCR and sequencing once the vector was isolated, as described in Section 2.14. This plasmid was then conjugated into the appropriate *Streptomyces* strain, as described in Section 2.11, and the ex-conjugants were subjected to biological activity and metabolic analysis, as described in Section 2.6. Although attempts were made to encourage the loss of the pCRISPomyces-2 plasmid, these experiments were conducted on strains that maintained the disruption plasmid due to time constraints. Despite this, complementation was conducted on the marked strains as per Section 2.16, which were also assessed for their biological activity.

5.2 Analysis of *Streptomyces* FG4 + pCRISP-2 *ctg_4838* Disruption Plasmid

Streptomyces FG4 + pCRISP-2 *ctg_4838* disruption plasmid (*S.* FG4+dp) was challenged by *B. subtilis, E. coli* and *C. albicans* on the same variety of media as described in Section 2.6, in triplicate. The wild-type had repeats of the bioassays performed alongside the ex-conjugant to act as a negative control, as were the complimented conjugants, where *ctg1_4838* had been restored. In all cases, the complementation restored the phenotype to that of the wild-type. The results of these bioassays, as presented in Table 49, demonstrated the complete loss of activity against *C. albicans* on all the media upon which the wild-type was active (SFM – shown in Figure 48, YP, YPD, SM25 and GYM+2µg/mL streptomycin). This indicates that it is

likely that Region 18 encoded for an antifungal agent able to inhibit the growth of *C. albicans* and that the deletion of *ctg1_4838* was able to sufficiently alter the biosynthetic pathway to disrupt biosynthesis. This was unexpected, as Region 18 was targeted for its homology to the Gram-negative inhibitor kanamycin, which does not possess antifungal activity. Further, Gram-negative activity was maintained across multiple media (FML, IMA, SM3, SM12, SM18, SM25, GYM+10µg/mL streptomycin), including gaining activity upon several which the wild-type did not demonstrate activity against *E. coli*. Although the ex-conjugant did loose *E.* coli activity on minimal, SM6 and minNAG. This suggests that regulation was affected more widely than was hoped when conducting a relatively small edit to the *Streptomyces* FG4 genome by targeting a single PKS. It is possible that Region 18 encoded two separate BGCs, one encoding for kanamycin and the other, one of which was knocked out by the CRISPR/Cas9 edit, encoding for an antifungal compound – but that antiSMASH determined these to be one cluster. This may also explain the changes in bioactivity against *E. coli* observed, given that a mutation close to the kanamycin BGC may impact the regulators of the kanamycin cluster. Further disproving the original hypothesis that Region 18 was one cluster with a PKS-based modification of kanamycin was the fact that *S.* FG4+dp was still able to inhibit kanamycin-resistant *E. coli*, similarly to how the wild-type demonstrated the ability to in Section 3.3.7. This suggests that the hypothecated modifications to kanamycin allowing it to inhibit *E. coli* pET28a do not involve the targeted PKS gene. This does not, however, exclude the possibility that the deactivation of Region 18, if it was a single BGC, did not result in the activation of an alternative antimicrobial-encoding BGC, which would also explain the change in the antimicrobial profile. That a different set of antimicrobial compounds are upregulated due to this change may also explain why the antimicrobial activity of *S.* FG4+dp against *B. subtilis* was also changed, with the ex-conjugant losing activity on SFM, SFMNAG, MYM, minimal, SM7, SM15 and minNAG but gaining it on IMA, SM18, GYM+2µg/mL streptomycin and ISP2. This gain and loss of anti-Gram-positive activity suggests that global regulation leading to a different smBGC being activated is the cause rather than a change in the compound produced by Region 18.

Figure 48 – Wild Type *Streptomyces* FG4 (left) and *S.* FG4+dp (right) when challenged by *C. albicans* on SFM. The wild type here inhibited *C. albicans* across the majority of the petri dish, whereas *C. albicans* could grow up to the *Streptomyces* FG4 + pCRISP-2 *ctg_4838* disruption plasmid colony. In the right-hand image, the *C. albicans* has grown as a confluent lawn across the plate, up to the actinomycete colony, which is seen as the consistent colour on the medium, which is slightly darker than the medium alone (not shown).

The inability for *S.* FG4+dp to grow on SFMSB, which the wild-type and complimented ex-conjugant can grow on, further supports the idea that global regulation was affected. Alternatively, it may be that *Streptomyces* FG4 requires Region 18 to survive when cultivated on sodium butyrate, although why that may be is unclear. It is possible that the compound Region 18 encodes is, in fact, primarily a regulator, not an antimicrobial, and that the butyric acid is up- or down-regulating a BGC to extremes when *ctg1_4838* is removed and is unable to regulate in opposition to butyric acid, leading to the cell being unable to survive. This would be similar to how some other antimicrobials have a significant signalling role at sub-inhibitory concentrations (Davies, Spiegelman and Yim, 2006; Andersson and Hughes, 2014). The idea that Region 18 encodes a compound with a significant regulatory function does help to explain the changes observed in *B. subtilis* and *E. coli* inhibition and may also explain the cessation of antifungal activity. If Region 18 encodes a regulator that upregulated the sole antifungal-encoding smBGC in the genome but is itself was encoding only a regulator, then knocking-out Region 18 would result in the loss of antifungal activity and, potentially, the change in antibacterial activity observed.

Given the wide range of phenotypical changes observed upon the deletion of *ctg1_4838,* the primary role Region 18's product in *Streptomyces* FG4 may be that of a signalling and

regulation molecule rather than an antimicrobial. It also is possible that Region 18 is comprised of two smBGCs, one encoding this regulator and the other a kanamycin derivative able to evade phosphorylation by KanR and thus inhibit *E. coli* pET28a. Alternatively, off-target mutations could have occurred in global regulatory genes or other antimicrobials, resulting in a broad range of phenotypic changes. The latter explanation seems more likely due to these phenotypic changes, however the structure of Region 18, discussed in Section 5.1, which lacked several genes found in the kanamycin smBGC but contained several others not found in the reference smBGC, means that is the possibility of the former explanation cannot be ruled out. Due to a lack of time, investigations into off-target mutations were not conducted.

Table 49 - Comparison of bioactivity between wild-type *Streptomyces* FG4 and *S.* FG4+dp. The exconjugant lost the ability to inhibit the growth of *C. albicans* across all media, suggesting that *ctg_4838* is required to produce the antifungal agent either as a regulator or as a core biosynthetic gene. The exconjugant did also lose the ability to survive on SFMSB, as well as demonstrating the ability to inhibit *B. subtilis* and *E. coli* on a different selection of media to the wild type and complimented strain. This may indicate that regulation was more widely impacted than was desired or expected. All replicates for each indicator strain are presented here in separate columns.

Actinomycete Strain		*S.* FG4+dp			Wild Type *Streptomyces* FG4 and Compliment		*S.* FG4+dp			Wild Type *Streptomyces* FG4 and Compliment		*S.* FG4+dp			Wild Type *Streptomyces* FG4 and Compliment	
Agar Name	**Target Strain**	***B. subtilis***					***E. coli***					***C. albicans***				
SFM		N	N	N	P	P	N	N	N	N	N	N	N	N	S	S
SFMNAG		N	N	N	S	S	N	N	N	N	N	N	N	N	N	N
SFMSB		F	F	F	N	N	F	F	F	N	N	F	F	F	N	N
YP		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
YPD		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SPY		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
FML		P	P	P	S	S	P	P	P	N	N	N	N	N	S	S
MYM		N	N	N	P	P	N	N	N	N	N	N	N	N	N	N
YEME		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
IMA		S	S	S	N	N	S	S	S	N	N	N	N	N	N	N
GYM		N	N	N	N	N	N	N	N	N	N	N	N	N	S	S
Minimal		N	N	N	S	S	N	N	N	P	P	N	N	N	S	S
SM3		P	P	P	P	P	P	P	P	N	N	N	N	N	N	N
SM5		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM6		P	P	P	P	P	N	N	N	P	P	N	N	N	N	N
SM7		N	N	N	S	S	N	N	N	N	N	N	N	N	N	N
SM12		S	S	S	S	S	S	S	P	P	P	N	N	N	S	N

SM14	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM15	N	N	N	S	S	N	N	N	N	N	N	N	N	N	N
SM18	P	P	P	N	N	P	P	P	N	N	N	N	N	N	N
SM19	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM20	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM25	P	N	N	N	N	P	P	P	N	N	N	N	N	N	N
SM30	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM32	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
MinNAG	N	N	N	P	P	N	N	N	S	S	N	N	N	S	S
GYM + 10µg/mL Streptomycin	P	P	P	S	S	P	P	P	P	P	N	N	N	N	N
GYM + 2µg/mL Streptomycin	P	P	P	N	N	N	N	N	N	N	N	N	N	S	S
YP/C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
ISP2	P	P	P	N	N	N	N	N	N	N	N	N	N	N	N
ISP4	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

5.3 Analysis of *Streptomyces* KY2 + pCRISP-2 *ctg_2919* Disruption Plasmid

Similarly to *S.* FG4+dp, *Streptomyces* KY2 + pCRISP-2 *ctg_2919* disruption plasmid (*S.* KY2+dp)was assessed for biological activity in triplicate as per Section 2.6 against *B. subtilis, E. coli* and *C. albicans.* The wild-type and complimented ex-conjugants were also assessed simultaneously, as presented in Table 50. No unmarked version of *S.* KY2+dp was generated, including after either 37 generations cultivated on solid media or 30 generations cultivated on solid media followed by a further four in liquid culture. *S.* KY2+dp maintained biological activity against all three indicator strains, although on a different selection of media as to the wild type. Against *B. subtilis,* the wild-type demonstrated bioactivity on SFM, SFMNAG, YP, YPD, SPY, YEME, GYM and SM18 where *S.* KY2+dp did not, instead demonstrating antimicrobial activity on Minimal, SM6, MinNAG, GYM+10µg/mL streptomycin, GYM+2µg/mL streptomycin and ISP2, unlike the wild-type. Wild-type *Streptomyces* KY2 demonstrated the ability to inhibit *E. coli* on YPD, unlike *S.* KY2+dp, which gained the ability to inhibit *E. coli* on FML, SM6, SM20, MinNAG and ISP2 – all media that also elicited *B. subtilis* inhibition in *S.* KY2+dp. Finally, *C. albicans* activity was lost from *S.* KY2+dp on SFMSB, YPD, YEME, GYM+10µg/mL streptomycin and ISP2 but gained on SM6 and YPC. Neither the wild-type nor ex-conjugant could successfully grow on SM30 or SM32. This broad change in antimicrobial activity against all indicator strains suggests that deleting *ctgl_2919* impacted the regulation of antimicrobials in *S.* KY2+dp as it is unlikely that this one gene is involved in the production of both anti-bacterial and anti-fungal agents. This could be due to other antimicrobials, whose BGCs less closely resemble those previously reported and were detected by antiSMASH, being activated when the product of Region 16 is not produced at high concentrations. This may lead to these alternative antimicrobials being produced on a limited selection of media as they may also require the stress factors those media inflict on the *Streptomyces* colony. Given the apparent coupling of *B. subtilis* and *E. coli* activity in *S.* KY2+dp but not in the wild-type or compliment strains, it may be that this new antimicrobial can inhibit Gram-negative bacteria as well as Gram-positive, unlike the product of Region 16, which was observed to inhibit *B. subtilis* independently of *E. coli*. However, if Region 16 encoded an antimicrobial with a higher MIC for *E. coli* than the newly produced compound but with a similar one for *B. subtilis,* this may be due to the concentrations produced rather than the properties of the compound. It is also possible that the fact that the ex-conjugant remained marked influenced the

antimicrobial activity of the strain, potentially by affecting gene regulation. Further, there is also the potential for off-target mutations, similar to *S.* FG4+dp, for which there was insufficient time to investigate.

Table 50 - Comparison of bioactivity between wild-type *Streptomyces* KY2 and *S.* KY2+dp. The exconjugant maintained its ability to inhibit all indicator strains on at least one medium, although the selection of media that this inhibition was observed was different to that of the wild type and complimented strain. This suggests that *ctg_2919* is involved in the regulation of antimicrobials. All replicates for each indicator strain are presented here in separate columns.

Actinomycete Strain		*S.* KY2+dp			Wild Type *Streptomyces* KY2 and Compliment		*S.* KY2+dp			Wild Type *Streptomyces* KY2 and Compliment		*S.* KY2+dp			Wild Type *Streptomyces* KY2 and Compliment	
Agar Name	Target Strain	*B. subtilis*					*E. coli*					*C. albicans*				
SFM		N	N	N	P	P	N	N	N	N	N	N	N	N	N	N
SFMNAG		N	N	N	P	P	N	N	N	N	N	N	N	N	N	N
SFMSB		P	P	P	P	P	P	P	P	P	P	N	N	N	P	P
YP		N	N	N	S	S	N	N	N	N	N	N	N	N	N	N
YPD		S	N	N	S	S	N	N	N	S	S	N	N	N	S	S
SPY		N	N	N	P	P	N	N	N	N	N	N	N	N	N	N
FML		P	P	N	P	P	P	P	N	N	N	N	N	N	N	N
MYM		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
YEME		N	N	P	S	S	S	S	S	S	S	N	N	N	S	S
IMA		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
GYM		N	N	N	P	P	N	N	N	N	N	S	N	N	S	N
Minimal		S	S	S	N	N	N	N	N	N	N	N	N	N	N	N
SM3		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM5		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM6		P	P	P	N	N	S	S	S	N	N	S	S	S	N	N
SM7		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM12		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM14		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

SM15	N	N	N	F	F	N	N	N	N	N	N	N	N	N	N
SM18	N	N	N	S	S	N	N	N	N	N	N	N	N	N	N
SM19	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
SM20	P	P	P	P	P	S	S	S	N	N	N	N	N	N	N
SM25	N	N	P	S	S	N	P	N	N	N	N	N	N	N	N
SM30	F	F	F	N	N	F	F	F	F	F	F	F	F	F	F
SM32	F	F	F	N	N	F	F	F	F	F	F	F	F	F	F
MinNAG	S	S	S	N	N	S	S	N	N	N	N	N	N	N	N
GYM + 10ug/mL streptomycin	S	S	S	N	N	S	S	S	S	S	N	N	N	P	P
GYM + 2ug/mL streptomycin	S	S	S	N	N	S	S	S	S	S	N	N	N	N	N
YP/C	N	N	N	N	N	N	N	N	N	N	P	P	P	N	N
ISP2	S	S	S	N	N	S	S	S	N	N	N	N	N	P	P
ISP4	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

5.4 Comparative Metabolomics of Wild Type and Ex-Conjugant *Streptomyces* Strains

Given the differences in antimicrobial activity observed between the wild-type and ex-conjugant for *Streptomyces* FG4 and KY2, comparative metabolomics was performed on both strains. For *Streptomyces* FG4, both the wild-type and ex-conjugant were grown on SM18 to identify the compound responsible for the antibacterial activity observed in the ex-conjugant but not in the wild-type, and on minimal media, for the inverse reason. *Streptomyces* KY2 and *S.* KY2+dp was cultivated upon SFM, minNAG and SM6. Extractions were then produced as described in Section 2.17 using dH_2O, methanol and ethyl acetate. After antimicrobial activity (or lack thereof) of the extracts had been confirmed by the method described in Section 2.22 to match the phenotype observed during cultivation, analysed via an Agilent 1260 Infinity II HPLC machine. The mass spectrometry and UV/Vis data produced were then analysed via Pairwise analysis on XCMS Online, using the HPLC/Q-TOF parameters to identify any changes in the chemical components of the extract (Gowda *et al.*, 2014). This data was also analysed using Agilent's own MassProfiler Professional software suite. Neither software package revealed a significant difference between the wild-type and ex-conjugant datasets for extractions performed under the same conditions for either strain. Given the changes in antimicrobial activity, this was unexpected. Similarly to the troubleshooting performed on the extractions generated in Section 4, the extractions from the wild-type and ex-conjugant strains were tested for acid sensitivity by placing them in 0.1% formic acid in acetonitrile solution at room temperature for 15 minutes and passed through a C18 Sep-Pack to determine if they were becoming bound to the HPLC column. Both of these methods are described in Sections 2.24 and 2.25, respectively. In all examples, the formic acid and control samples maintained activity (or lack thereof) and antimicrobial activity was identified in the acetonitrile fraction (where appropriate). Thus, it seems unlikely that the compound is affected by the formic acid or is becoming stuck on the C-18 column inside the HPLC instrument. It is possible that these results are due to an error conducted during sample preparation, for example if two wild-type samples were used in place of one wild-type and one

ex-conjugant. Alternatively, it may be that concentrations were calculated and maintained incorrectly across experimental stages, resulting in a dilution of the antimicrobial below to MIC.

5.5 Discussion

Both *Streptomyces* strains analysed here proved compatible with the pCRISPomyces-2 plasmid. This is not the case for all *Streptomyces* strains, especially given that established protocols are designed in the model organism *S. lividans* 66 (Cobb, Wang and Zhao, 2015). Given that the strains modified here were isolated from fungus-growing ant colonies and have not previously been studied, they possibly could have proven resistant to modification by the pCRISPomyces-2. This may be for a variety of reasons; they may have fewer RNA polymerases or RNA polymerases not targeting the same areas as Cas9, which would limit the efficiency of Cas9 by blocking the removal of Cas9 from the DNA. This, in turn, would block DNA repair proteins from accessing the Cas9-generated breaks and prevent the Cas9 protein from causing double-strand breaks elsewhere (Clarke *et al.*, 2018). Alternatively, the strains might favour non-homologous end joining over homology-directly repair, which may affect the efficiency of the DNA repairing as desired for the mutation. Alternatively, they may have proven resistant to the antibiotics used in the CRISPR/Cas9 protocol, as observed in several other actinomycete strains studied here. Already having resistance to the antibiotic used for the selection of plasmids limits that antibiotic's ability to select colonies containing that plasmid – this, in turn, makes it difficult to determine which colonies surviving on a petri dish should be selected for onward processing and which have expelled the plasmid. Developing new plasmids with alternative antibiotics would be possible, although this may need significant time and optimisation. That CRISPR/Cas9 was successful also demonstrated the ability to conjugate into *Streptomyces* FG4 and KY2 – which is not universally true of all *Streptomyces* strains. There are two features of many *Streptomyces* species which limit their receptiveness to complementation. Firstly, many *Streptomyces* strains utilise methyl-specific restriction mechanisms to protect against the introduction of heterologous DNA, for example, from phage (MacNeil, 1988; González-Cerón, Miranda-Olivares and Servín-González, 2009). In this study, methylation deficient *E.*

coli ET12567/pUZ8002 was used as a donor, although it would be interesting to determine if the *Streptomyces* strain studied here would accept DNA from DH5α pUZ8002, where methylation is present, given the relative difficultly in cultivating *E. coli* ET12567/pUZ8002. Further, all conjugations into *Streptomyces* strains must contain the origin sequence *oriC* – from which chromosomal replication is initiated. If this sequence is lacking, the inserted vector will not be replicated (Jakimowicz *et al.*, 1998; Hopwood, 2006). Given that *oriC* sequences can vary, it would be feasible that the *oriC* sequence in pCRISPomyces-2 would not be recognised by the receiving bacterium. Further success is that complementations of both strains were successfully generated and that the phenotype was restored to that of the wild-types. This demonstrates that the available protocols can be applied to future attempts to genetically manipulate *Streptomyces* FG4 and KY2 and that pre-existing vectors are compatible with these strains, opening the potential for further investigations into the antimicrobials they produce.

The CRISPR/Cas9 knockouts of Region 18 of *Streptomyces* FG4 and Region 16 of *Streptomyces* KY2 appeared successful, with some limitations. In both cases, antibacterial activity was gained on some media and lost on others, suggesting that regulators outside the targeted BGC were affected. This is even though, in both cases, the smallest edit to the BGC was made to prevent biosynthesis but minimise the effect on the wider organism. In *S.* FG4+dp, this resulted in complete loss of antifungal activity. Comparative metabolomics was performed between the wild type and ex-conjugant of both strains across multiple media in each case, but this revealed no difference in the contents of the extract. This is despite extracts taken from phenotypes demonstrating antimicrobial activity still processing that activity before and after HPLC analysis. This does not preclude this antimicrobial activity from being caused by a siderophore that is disrupted during the HPLC process but is not during the extraction and bioassay processes. It is also possible that both strains produce a collection of compounds which combine to present the antimicrobial activity observed. For *S.* FG4+dp, it seems unlikely that this is the case for the antifungal activity observed due to its cessation after *ctg_4838* was knocked-out, implying that Region 18 encoded for the antifungal in question. Disappointingly, it

was not possible to determine the structure of this antifungal. Further work should be conducted to optimise the extraction to increase yields and to determine why the antifungal activity of the extract is lost after HPLC analysis. It would appear unlikely that the compound responsible is a siderophore, given that antifungal activity was still observed upon iron-supplemented media and the homology between Region 18 and the kanamycin-encoding BGC. This same homology is, however, surprising given that kanamycin is an antibiotic rather than an antifungal agent. Region 18 may be two BGCs – one encoding kanamycin and the other encoding an antifungal compound but antiSMASH was unable to resolve the separation between them. This would explain the loss in antifungal activity but not why the extractions lost activity upon HPLC analysis; however, it is not clear that *Streptomyces* FG4 encodes the genes missing from the kanamycin smBGC unless they are present in a distinct part of the genome to the gene detected here. Further work is needed to investigate this possibility and to extract the potential kanamycin-like believed to be encoded within the genome; this is of interest given the ability for the compound to inhibit kanamycin-resistant *E. coli*.

In *S.* KY2+dp, it was also observed that a broad range of antimicrobial ability was impacted when *ctgl_2919* was deleted, although the maintenance of the pCRISPomyces-2 plasmid across multiple generations does limit the ability to draw conclusions. What was observed was a change in antimicrobial phenotype, with indicator strains inhibited on a different variety of media by the conjugant than the wild-type. Activity was maintained against all indicator strains by the ex-conjugant on at least one medium, although the media this was exhibited upon was different from those of wild-type and complimented strains that displayed antimicrobial activity. Further work is needed to understand this and to determine what role *ctgl_2919,* and Region 16 more widely, has within *Streptomyces* KY2. Although comparative metabolomics did not reveal any differences in extractions made from wild-type and ex-conjugant, this should be investigated further, perhaps across a wider variety of media or with further optimisation of extraction conditions to increase yields. Comparison of the extracts generated from *Streptomyces* FG4 and *S.* FG4+dp can be compared with each other, as can those generated from

Streptomyces KY2 and *S.* KY2+dp, however comparison between *Streptomyces* KY2 (and its associated ex-conjugant) and *Streptomyces* FG4 (and it associated ex-conjugant) cannot. The concentrations of the extracts were not only standardised to those they were being directly compared with, not those of the other strain.

To better understand the changes in antimicrobial activity observed, it would be worth identifying off-target mutations that may have occurred in either strain. These may be caused by the 20-nucleotide targeting sequence having high homology to other sections of the genome (Zhang *et al.*, 2015). Additionally, it has been shown in human cells that plasmid-delivered *Cas9* and sgRNA can lead to off-target DNA cleavage immediately after insertion (Ramakrishna *et al.*, 2014). If this is also true for *Streptomyces* species, it may help to explain some off-target mutations observed. To determine if these off-target mutations have occurred, the genomes of the mutant could be sequenced and compared to the wild-type. When sequencing, exome sequencing could reduce cost compared to whole genome sequencing and allows for non-bias identification of mutations in coding regions of the genome, however if mutations in non-coding regions had occurred they would not be detected. Alternatively, given the most common reason for off-target mutations is when a double-strand break is repaired by non-homologous end joining instead of homology-directed repair, direct *in situ* break labelling, enrichment on streptavidin, and next-generation sequencing (BLESS) could be performed. BLESS labels DSBs by flagging them with the antibody biotin and capturing these with streptavidin. This allows for BLESS to label the DSB, which may indicate where mutations have occurred however, it can only detect where current DSBs are located, not those that have been repaired (Zischewski, Fischer and Bortesi, 2017). Despite these potential alternatives, given that there are full genome sequences of the wild-type of these strains to use as references, it is probably optimal to perform full genome Sanger sequencing of the mutants to identify every mutation that may have occurred without bias. If off-target mutations are found, it would be worth investigating if these other mutations may explain the changes in phenotype observed, particularly if they are affecting other smBGCs with low homology to any previously-reported BGC, which may imply they are novel antimicrobials. Off-target mutations would not, however, explain the

lack of differences observed when comparative metabolomics was performed. These may be due to the extraction techniques not generating high enough yields of compound to be detected, although this would be surprising given their antimicrobial properties. It may be that a normal-phase HPLC would be better suited for analysing the samples in question, which may be more effective than the reverse-phase used here if the compound is lipophilic. Additionally, analysis using LC/MS/MS would allow for better identification of the compounds contained within the extracts and may uncover more differences between the conjugant and wild-type strains than LCMS.

6 Conclusions and further work

6.1 Investigate the Other Fungus-Growing Ant Associated Actinomycete Strains for Novel Antimicrobials

The original aim of this work was to determine the antimicrobial potential of the 18 ant-associated actinomycete strains that had previously been isolated and to determine if any of these had novel mechanisms of action. Pleiotropic techniques have been successfully deployed here to elicit biological activity from all 18 actinomycete strains against *B. subtilis* and *C. albicans,* with 10 showing the ability to inhibit *E. coli* on at least one of the 31 different media they were cultivated on. This does not mean that the remaining eight strains cannot inhibit *E. coli,* only that inhibition was not observed – it is possible that if more varieties of media were used, *E. coli* activity could be elicited. Eleven strains tested here did not express antimicrobial activity when grown on traditional actinomycete growth media – SFM, ISP2 and ISP4, instead requiring a more unusual media composition. This shows that pleiotropic methods, whilst not as targeted as more recent genetic-based techniques, are still effective at unlocking cryptic smBGCs. FML and SM25 media proved the most effective of those used here at activating antimicrobial smBGCs that were cryptic when the strains were cultivated on traditional growth medium, suggesting they should be prioritised when pleiotropic studies are performed on other actinomycetes. It would be interesting to see if these media are as effective at eliciting antimicrobial activity from strains that have not been isolated from fungal-growing ant colonies. If further work was to be conducted and was to elicit the ability to inhibit *E. coli,* then it may be more likely that this antimicrobial produced has not been previously reported. However, this may not be the case if a different, previously studied strain produced the same antimicrobial more readily. Therefore, it is probable that there is limited benefit to continuing pleiotropic techniques in an attempt to elicit the ability to inhibit *E. coli* in those strains that did not demonstrate the ability to during this work. Instead, time may be better spent interrogating the genomes, which will be discussed below in Section 6.3. That all the strains demonstrated antimicrobial ability suggests that fungus-growing ant colonies may be a potential source of novel antimicrobials if the strains can be studied effectively, as only a select few of the strains in this work were able to be investigated thoroughly – three by chemical extraction and two using genetic manipulation. Further investigations into the other actinomycetes may prove fruitful in the search for novel antimicrobials. In this work, it was

assumed that if a known antimicrobial was encoded in the genome, it was responsible for any observed antimicrobial activity, but this is not always the case. By attempting chemical extractions from all 18 strains from media upon which they demonstrated antimicrobial activity and, where relevant, comparing these extracts to reference samples of any antimicrobials known the be encoded within the genome, one could identify produced novel antimicrobials even if a strain contains a previously-reported antimicrobial encoding smBGC. If the previously-reported antimicrobial is not the one being produced by the strain, then there will be no peak corresponding to the reference standard. Work can then be performed to determine which compounds in the extract are potentially novel antimicrobials. Investigations into the rare actinomycetes may prove especially fruitful, as fewer *Amycolatopsis*, *Agrococcus, Tsukamurella* and *Jiangella* genomes have been reported previously. This relative lack of study means that not only may they encode antimicrobials that are extremely rare outside of their species, but the bioinformatics tools, such as antiSMASH, may be less able to identify smBGCs encoded in these strains. It may be that these strains structure their smBGCs differently to *Streptomyces*, for example, by using different, previously unreported regulators, which may impact the ability for antiSMASH to identify them.

That all strains studied here exhibited antifungal activity provides evidence to support the theory that these strains are recruited from the environment around them to assist with the protection of the fungal garden from invasive *Escovopsis* species (Barke *et al.*, 2010). Samples from more fungus-growing ant colonies could help build this hypothesis, but a better evidence base could be built if the ants were exposed to soil that had been sterilised and then inoculated with actinomycetes known to produce antifungal agents or known not to. This could then provide some information on the ant's ability to recruit antimicrobial-producing actinomycetes over actinomycetes more generally. This would be of interest in increasing our understanding of the ant-actinomycete relationship, the chemical ecology of the nest and the formation of the ant microbiome and would also demonstrate if fungus-growing ant colonies are a potential reservoir of previously undiscovered antimicrobials. It would also be of interest to investigate how the antimicrobial resistance profile of the fungal garden is affected by changes in the actinomycetes present in the colony – it would be undesirable for the ants to carry an actinomycete that is producing an antifungal agent capable of inhibiting their

cultivar. This may be challenging, however, due to the difficulty of cultivating the fungal cultivars outside of their natural environments, especially if plasmids drive resistance and the bacterium readily sheds such plasmids.

6.2 Characterise the Antimicrobials Extracted from *Streptomyces* FG4 and KY2 and *Amycolatopsis* UM15

Extractions with antimicrobial activity were successfully produced from *Streptomyces* FG4 and KY2 and *Amycolatopsis* UM15. However, there was insufficient time to purify these extracts. This is despite multiple attempts and a series of troubleshooting steps to optimise the HPLC and ensure the sample was not adversely interacting with the instrument. More time being spent on the purification and then identification of the antimicrobial compounds extracted would be highly desirable. Normal-phase HPLC may allow for better resolution of the antimicrobial compound, but it may also be that multiple compounds working in tandem are responsible for the observed inhibition. Additionally, alternate mobile phases to the acetonitrile and methanol used here could be used, such as water, as well as alternative stationary phases, such as C5, if it is suspected that the antimicrobial activity is due to a macromolecule, such as a lanthipeptide. Once purified, the structures of the antimicrobials should be characterised using ^{1}HNMR, ^{13}NMR and LC/MS/MS. COSY NMR can also be employed if a better understanding of proton coupling is required. The compound's infra-red spectrum may also help identify functional groups, especially aromatic rings. This data can then be analysed with tools like the Global Natural Products Social Molecular Networking infrastructure to identify unknown parent ions (M. Wang *et al.*, 2016). Determining the structure of the compound allows for predictions of its mechanism of action and potentially assists in determining which smBGC may be encoding it. Predictions of the biosynthetic pathways of the purified compounds would allow effective targeting of smBGCs that appear able to encode that pathway with CRISPR/cas9 to observe the impact on their metabolome and antimicrobial activity. Disabling antimicrobial biosynthesis would also allow comparative metabolomics and the biosynthetic pathway to be fully characterised.

6.3 Expand Genome Mining Using CRISPR/Cas9

Ex-conjugants of two environmental *Streptomyces* strains were successfully produced using the pCRISPomyces-2 system and were observed to present a different phenotype to the wild-

type. For one strain, *Streptomyces* FG4, there was a complete cessation of antifungal expression. These ex-conjugants were then successfully complimented, restoring the original phenotype. This demonstrates the ability for existing genetic modification techniques to be utilised on a wider range of non-model organisms and opens the potential for further investigations of these strains using the pCRISPomyces-2 vector. It seems likely that an smBGC encoding an antifungal agent was successfully targeted, opening the possibility of further genetic modification being used to improve our understanding of the targeted region, including the option for targeting other genes in the biosynthetic pathway as a way of deducing the structure of the product. It would also be of interest to attempt to heterologously express the smBGC in *E. coli*, to allow for potentially higher yields if extractions are attempted and for easier further study of the smBGC due to the faster growth of *E. coli* and the relative ease of genetic modification in *E. coli.* For *Streptomyces* KY2, although the CRISPR/cas9 modification was successful, it did not eliminate antimicrobial activity. Instead, the media upon which activity was observed changed, suggesting that the targeted region was more likely to be involved in regulation than encoding an antimicrobial itself, or, if the region did encode an antimicrobial, it activated others in the genome. Further study would be needed to determine which is more likely. Despite this success, comparative metabolomics of both strains failed to determine any significant difference between extractions from the wild-type and ex-conjugants. It may be that the antimicrobial is highly potent, so is only produced in small concentrations and thus require highly sensitive instruments and difficulty separating the signal from background noise in the sample. Heterologous expression, if successful, may assist with this by increasing yields, as would creating more extracts from the *Streptomyces* strains – although this would be labour intensive. It is also possible that optimisation of the HPLC analysis would improve the ability to detect any differences between the extracts. Optimisation of the UV detector's wavelengths may help with detection, and alternative liquid phases may improve resolution.

Other than *Streptomyces* FG4 and KY2, CRISPR/cas9 modification was not attempted on the strains in this study. This work did, however, characterise some important attributes of the 18 strains for future attempts at genetic manipulation, finding that 10 of the strains were susceptible to the antimicrobial resistant markers utilised in the pCRISPomyces-2 system and determining the incubation time required for these strains to sporulate – which was

confirmed using SEM. This lays the groundwork for future attempts at genetic modification of these strains. This could be of interest not only to develop our knowledge of the limits of the pCRISPomyces system in environmental isolates but also to determine its applicability to rare actinomycetes. In addition to sporulating on SFM media, three *Pseudonocardia* strains (UM4, UM14 and UM9), *Agrococcus* A6 and both *Tsukamurella* FG11 and E7 strains proved susceptible to both apramycin and hygromycin, potentially allowing for the pCRISPomyces-2 system to either be applied to them. This may require modification of the pCRISPomyces-2 vector, for instance, if the strain uses alternative *Ori* sequences to *Streptomyces* species or if the strains are challenging to conjugate into. There is potential, however, for a vast array of bacteria to be investigated in more depth if pCRISPomyces-2 can be applied to these rare actinomycetes. The six strains that demonstrated susceptibility to the antibiotics used in pCRISPomyces-2 contained 51 smBGC with low homology to previously-reported smBGCs, with a vast array more smBGCs in other rare actinomycetes that a hypothetical pCRISPomyces-3 may be compatible with, improving the ability to interrogate those smBGC whose function is currently unknown.

More interrogation of the smBGCs encoded in the 18 strains studied here could be performed using bioinformatic tools like Biosynthetic Gene Similarity Clustering and Prospecting Engine (BiGSCAPE) and CORe Analysis of Syntenic Orthologs to prioritize Natural Product Biosynthetic Gene Clusters (CORASON). These tools would reveal the relatedness of the smBGCs encoded within the strains assessed here, both with each other and previously-reported antimicrobial encoding smBGCs. This could provide more information as to the potential of these strains to encode novel antimicrobials and may provide further information as to how the ants select non-*Pseudonocardia* actinomycetes and if there is any evidence of horizontal transmission. They may also provide some information about the evolutionary history of the smBGCs encoded in the strains studied here. These tools may also reveal if an smBGC is duplicated between the strains presented in this work, which may assist in targeting antimicrobial encoding smBGCs if multiple strains demonstrating similar antimicrobial profiles all contain the same smBGC that is not otherwise conserved. Antibiotic Resistant Target Seeker could also be used to identify smBGCs that are more likely to be encoding antibiotics, especially those with novel targets (Alanjary *et al.*, 2017). These smBGCs could then be targeted for

manipulation using CRISPR/cas9 or for heterologous expression in *E. coli* to determine the products they encode.

It would be interesting, both in terms of improving our understanding of the ant microbiome and for identifying potentially novel antimicrobials, to uncover which smBGCs are being expressed when the bacteria are living on the ant carapace. RNA-Seq from a strain growing on the ant carapace could be compared to RNA-Seq data generated from that same strain cultivated on a medium upon which it expresses antimicrobial activity. This would narrow down the potential targets for CRISPR/cas9 knock-outs by predicting the biosynthetic pathway of the purified compound, then targeting smBGCs that are being expressed, but the function for their products is unknown.

6.4 Final Conclusions

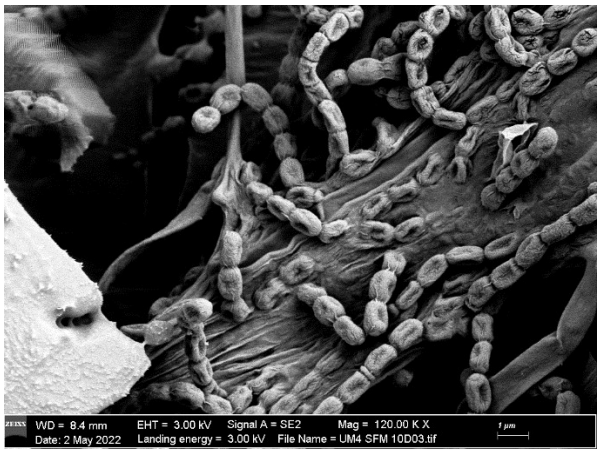
The work presented here demonstrates the potential for actinomycetes isolated from fungal-growing ant colonies to possess smBGCs encoding novel antimicrobials. Pleiotropic techniques have been demonstrated to be effective at eliciting antimicrobial activity from otherwise cryptic smBGCs. By comparing the observed biological activity with antiSMASH predictions of the smBGCs present in the genomes, it appears likely that some of these antimicrobials are novel. Extractions of antimicrobials from three strains were successfully conducted, although they were not purified. Further work to purify and characterise the extracted antimicrobials should be prioritised in the hopes of discovering an antimicrobial with a novel mechanism of action. Although ex-conjugant from two strains – *Streptomyces* FG4 and KY2 - were generated using the pCRISPomyces-2 vector, the pCRISPomyces-2 plasmid was well-retained by both strains. Cultivating further generations in liquid and solid cultures may allow for the loss of this plasmid. Complementation of the ex-conjugants was successfully performed, restoring the wild-type phenotype and demonstrating the ability to use existing techniques to interrogate the genomes of these strains. Further genetic studies could be conducted on the other strains in this study, targeting smBGCs suspected of encoding antimicrobials, which could develop our understanding of how environmental strains respond to attempts to modify them using the CRISPR/cas9. These studies may also reveal other antimicrobials not expressed when the strains were analysed with pleiotropic techniques.

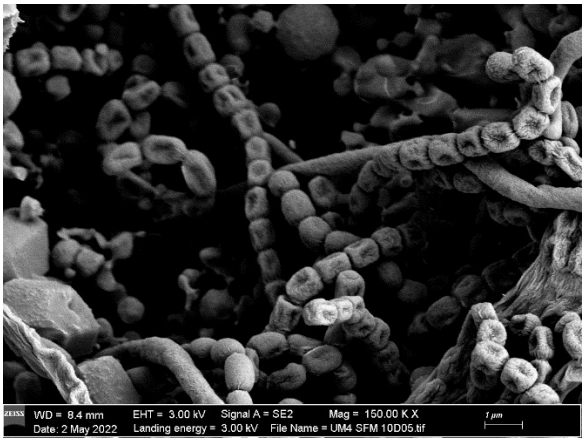
In conclusion, the work presented here represents a first look at the 18 actinomycete strains isolated from fungus-growing ant colonies. It reveals the biosynthetic potential of these strains and provides a launch pad from which further study of these strains can be conducted.

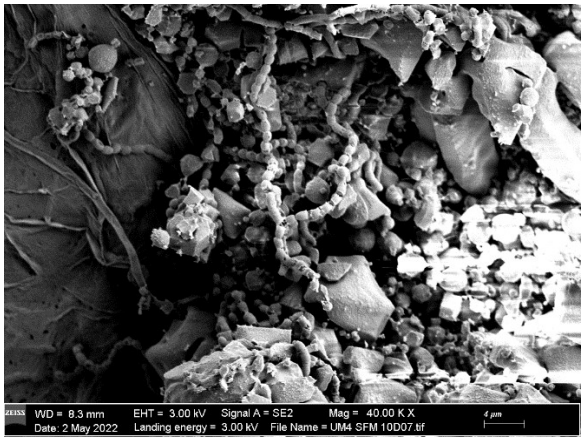
7 Appendix

7.1 SEM Images

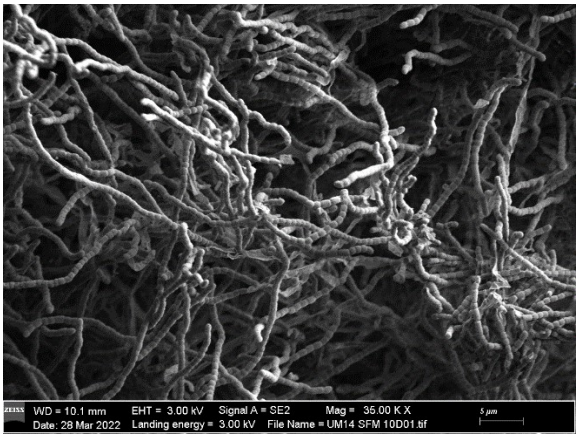
7.1.1 *Pseudonocardia* UM4

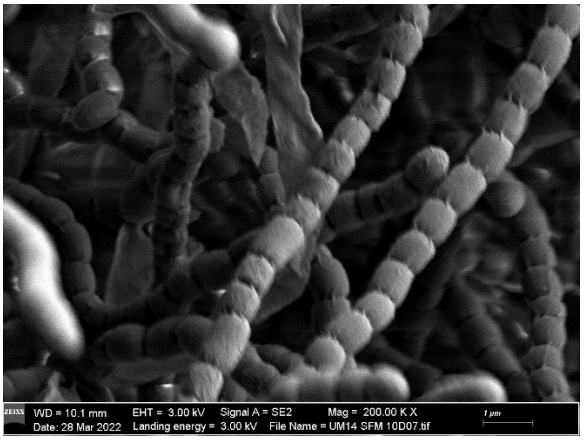






7.1.2 *Pseudonocardia* UM14

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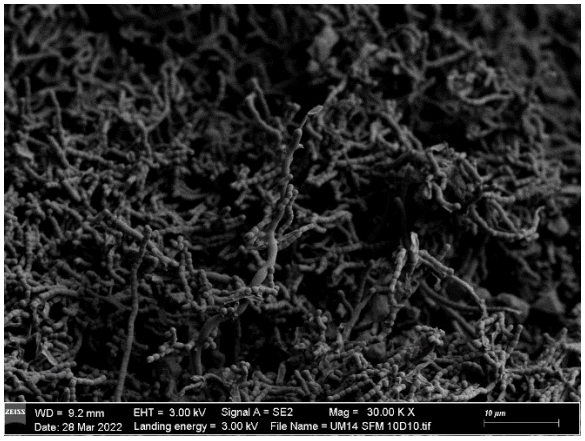
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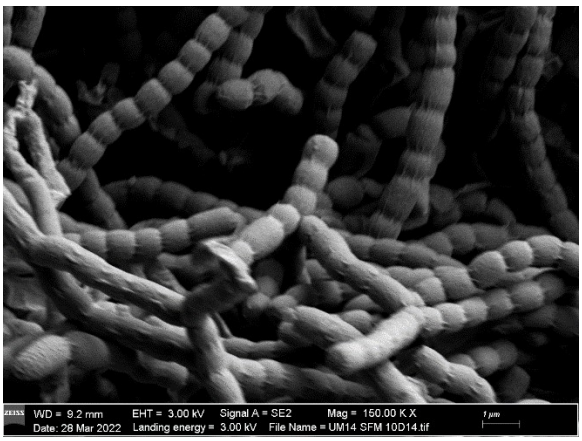
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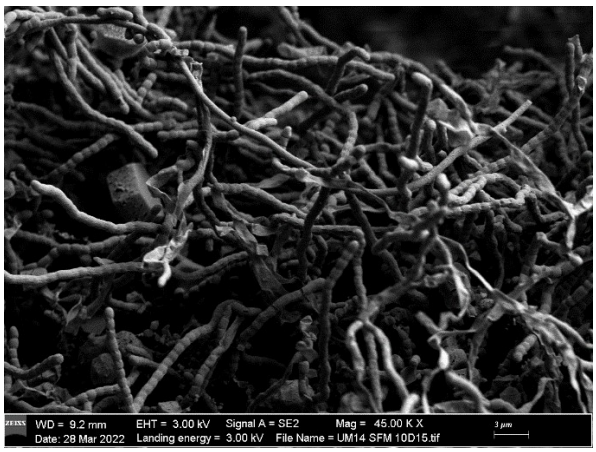
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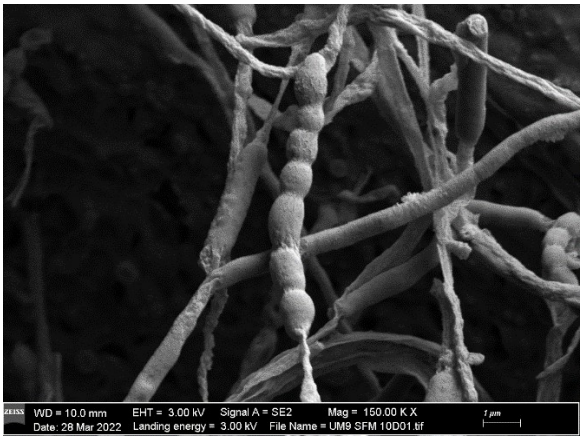
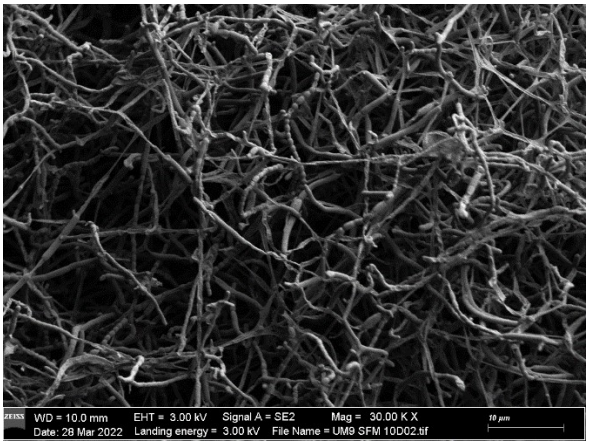
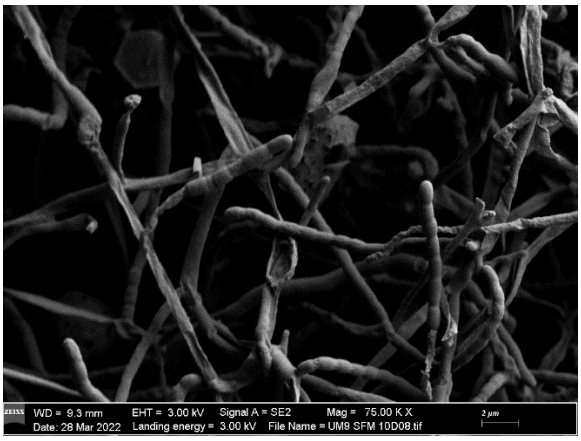
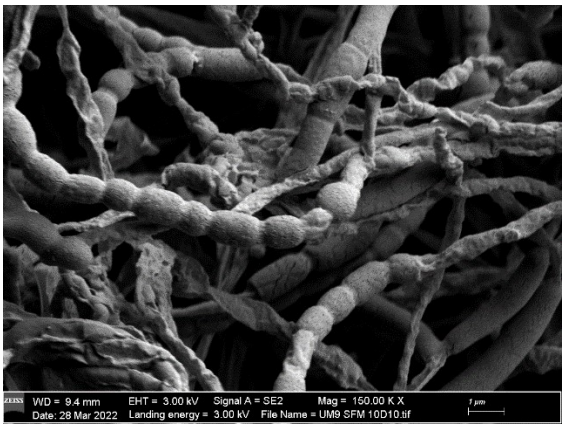
3 µm

7.1.3 *Pseudonocardia* UM9

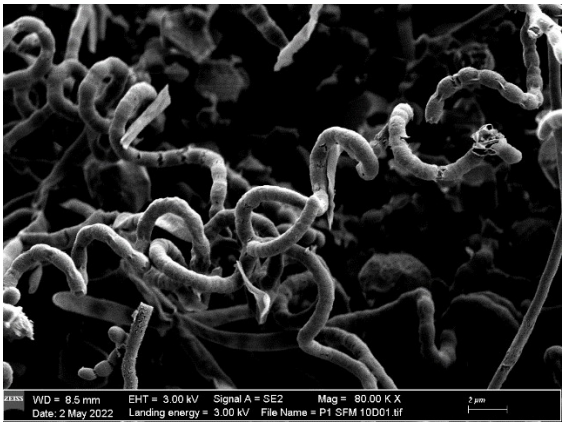


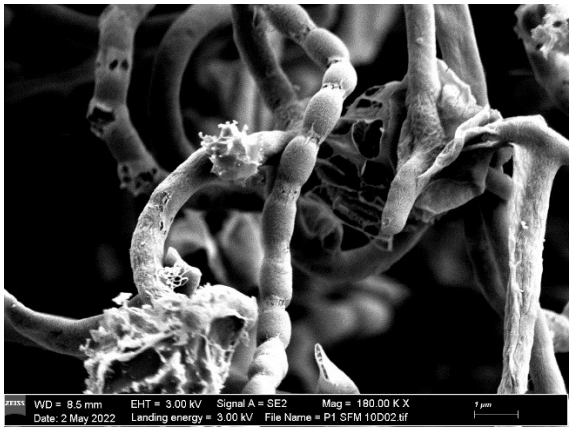


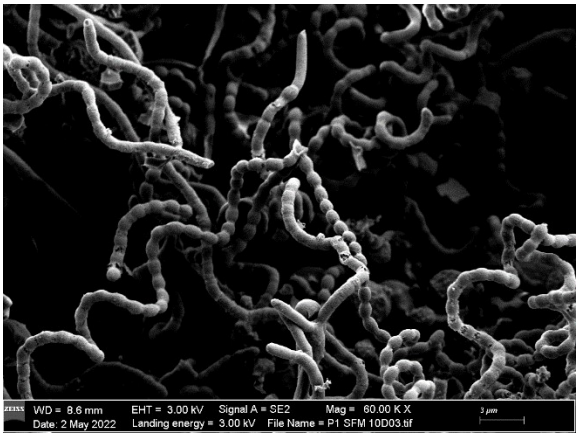


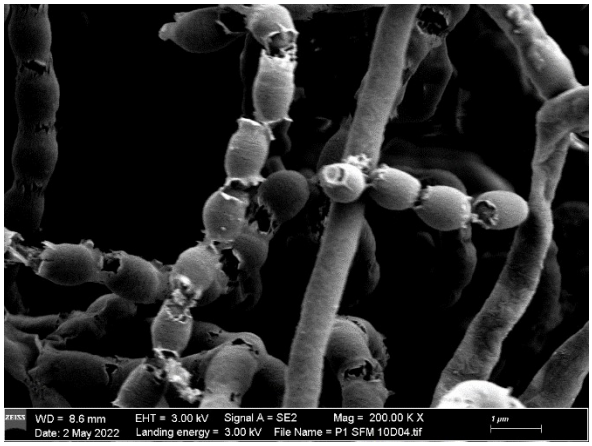


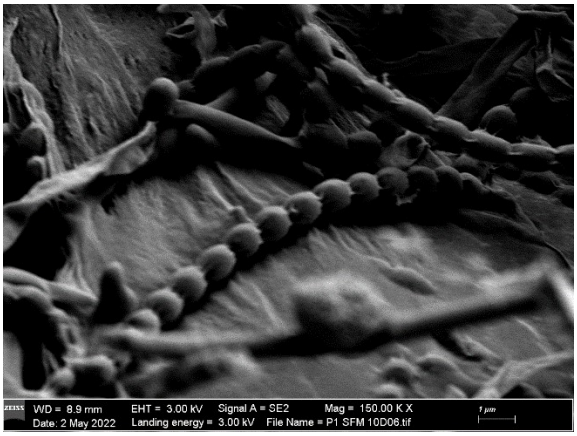
7.1.4 *Pseudonocardia* P1



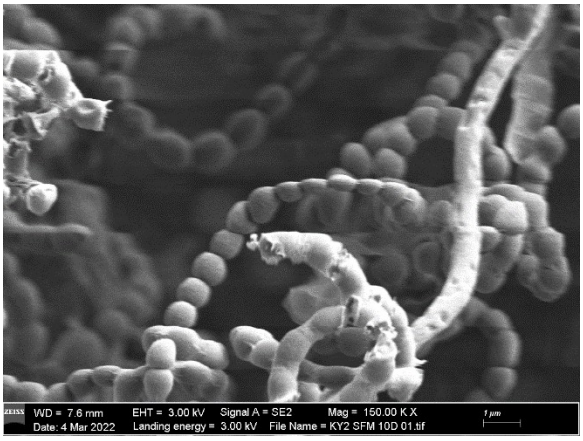


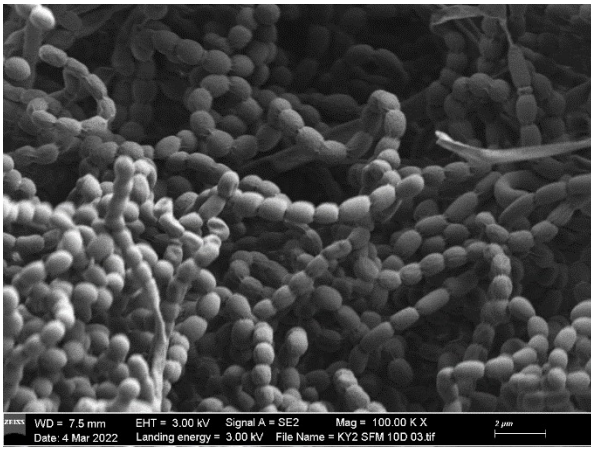


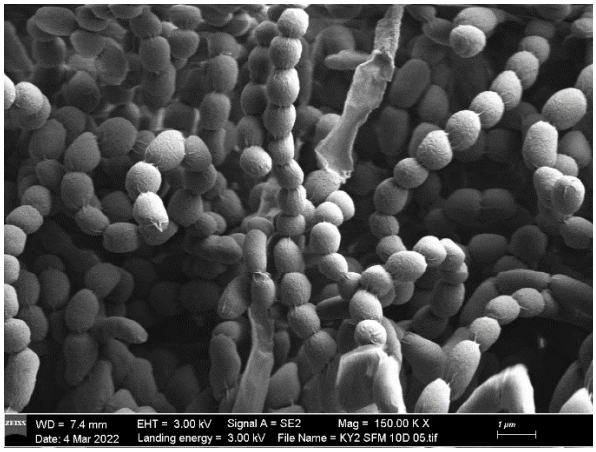


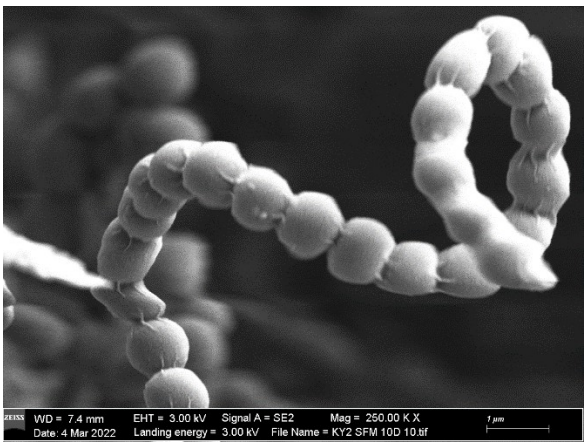


7.1.5 *Streptomyces* KY2

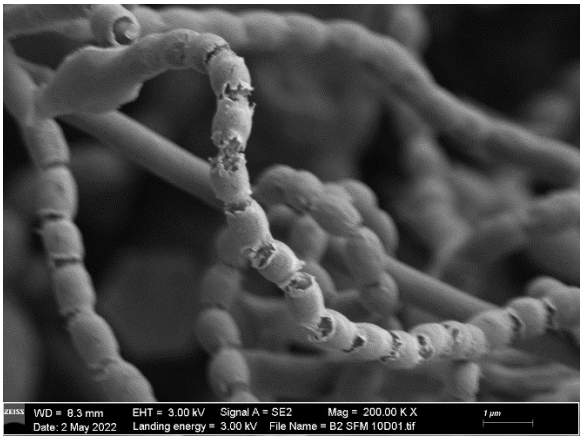


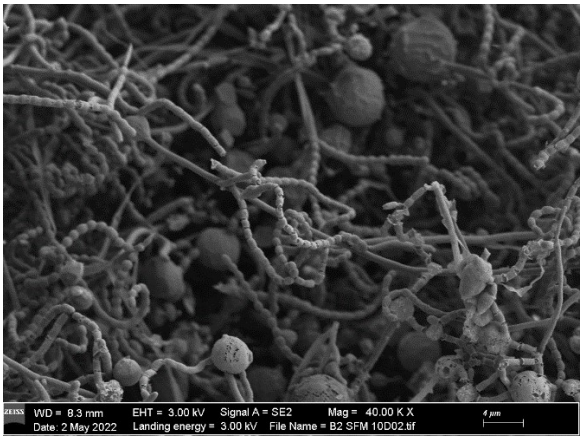


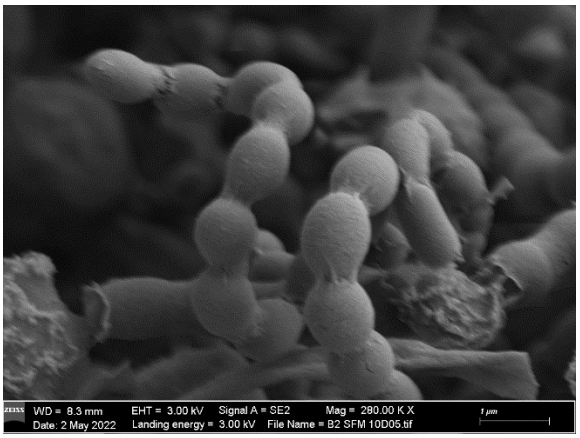


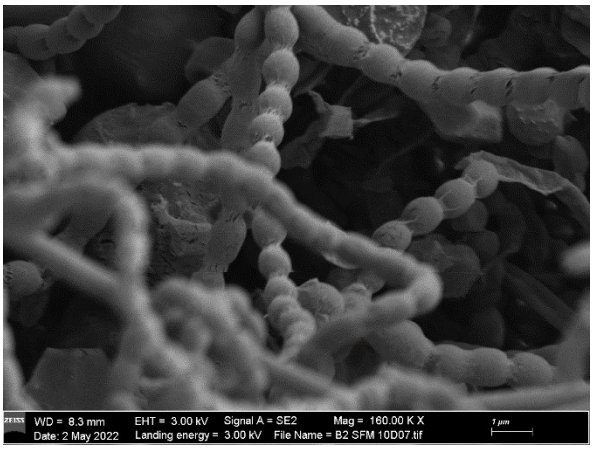


7.1.6 *Streptomyces* B2

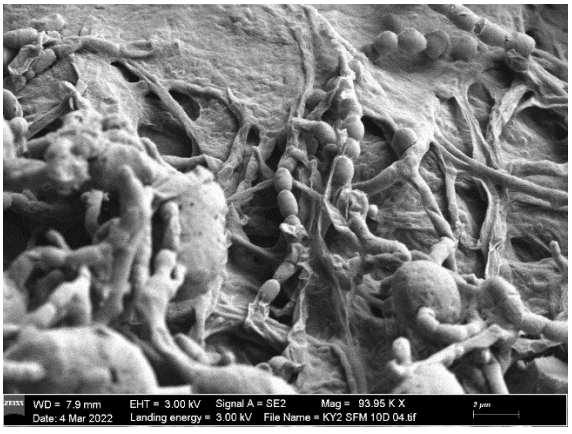


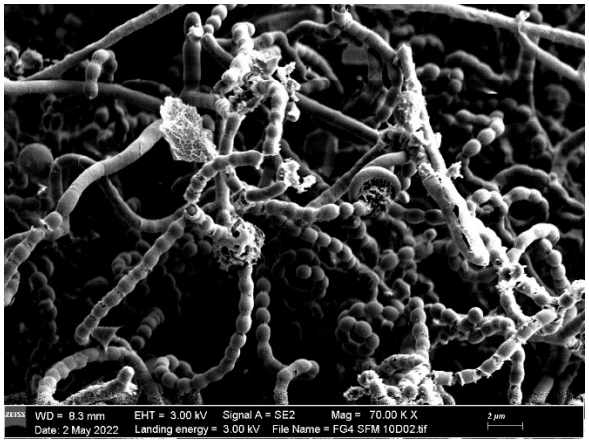


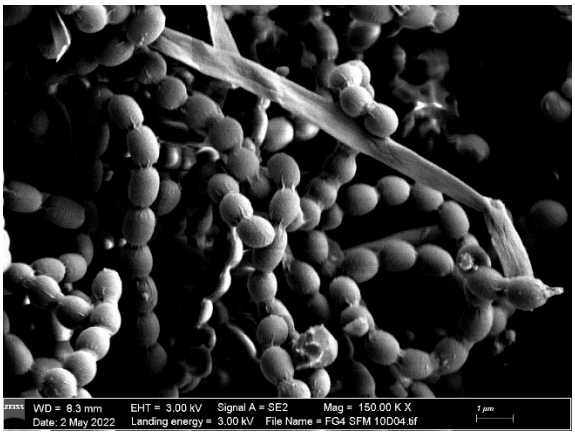




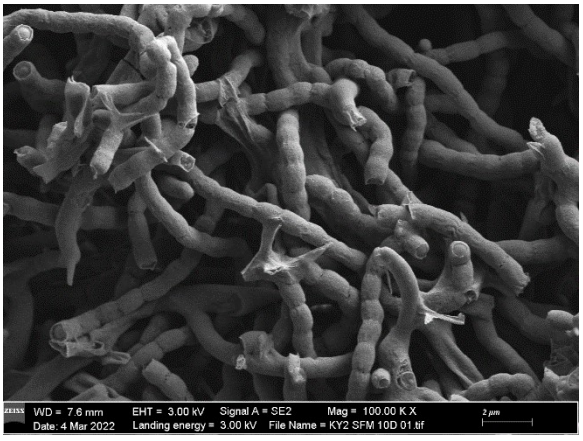
7.1.7 *Streptomyces* FG4

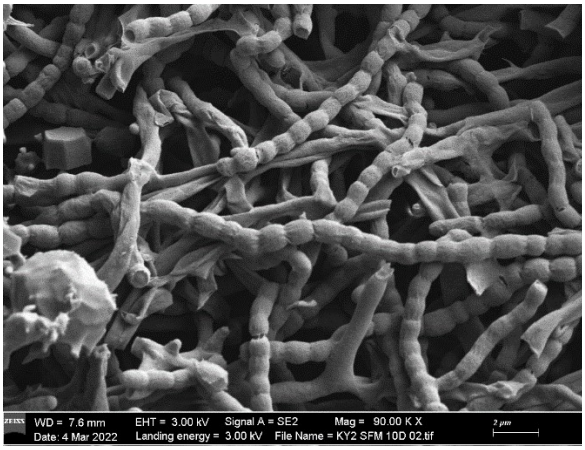


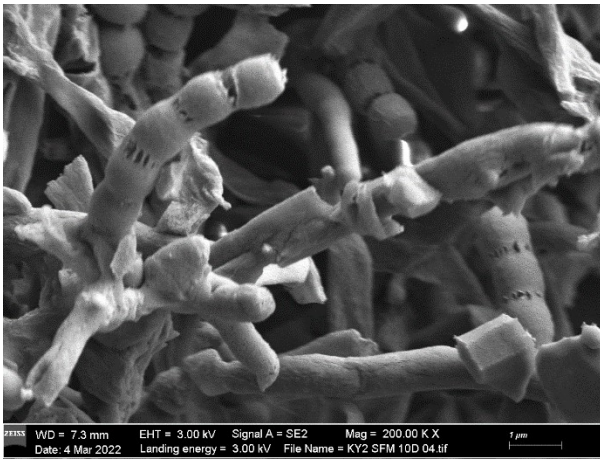


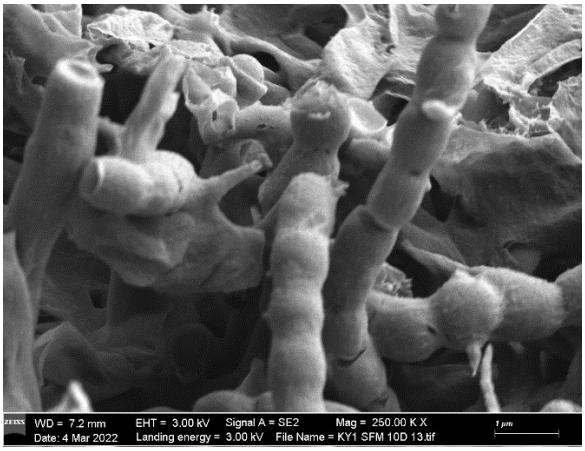


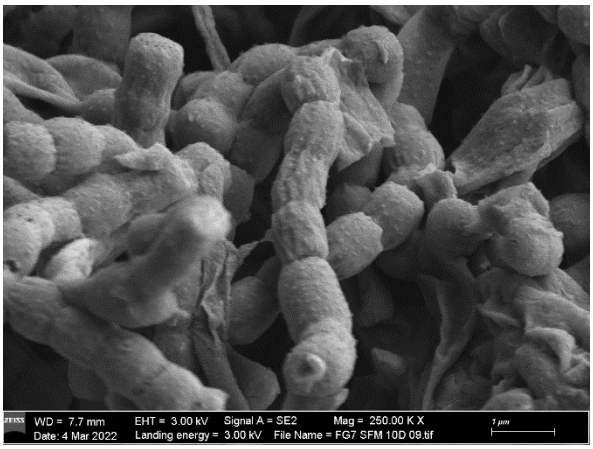
7.1.8 *Streptomyces* FG7

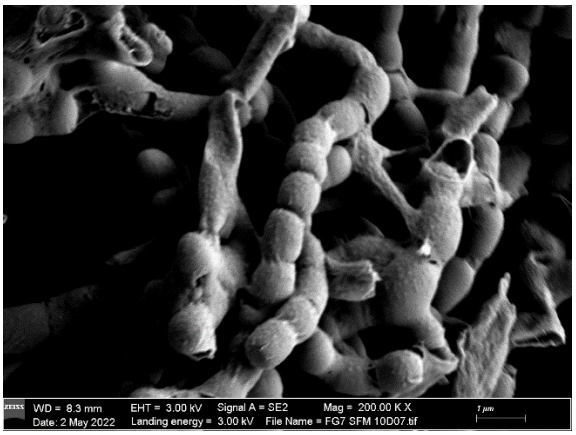




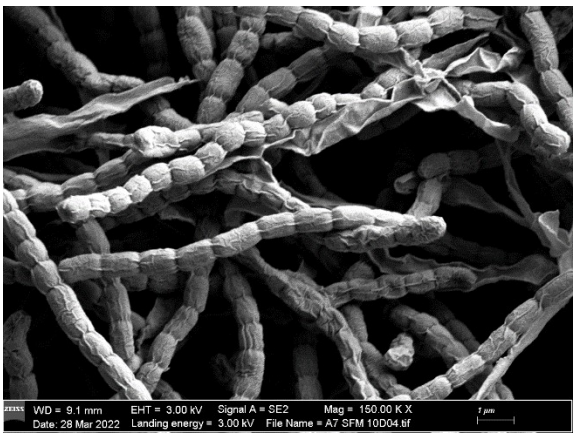


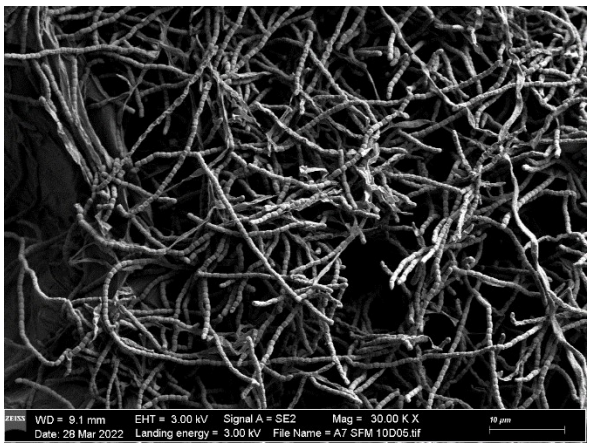


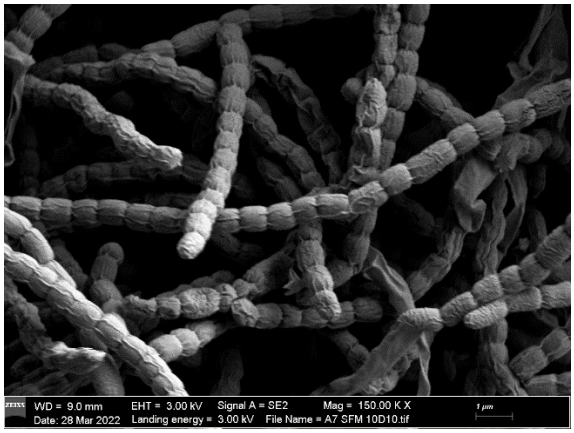


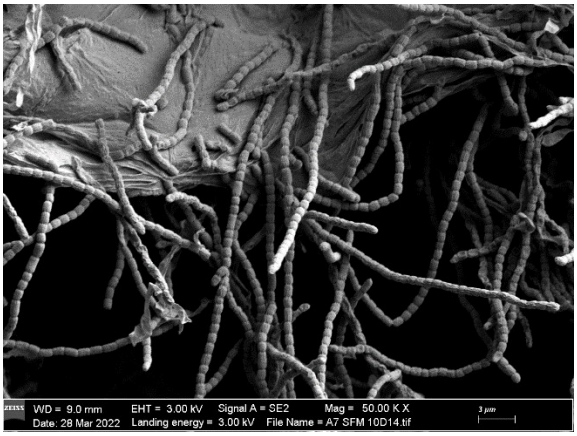


7.1.9 *Streptomyces* A7

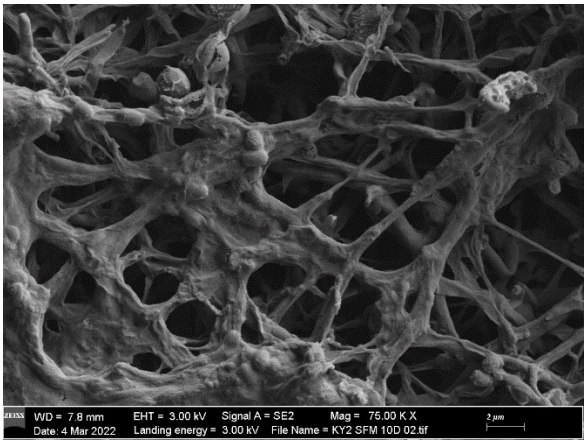


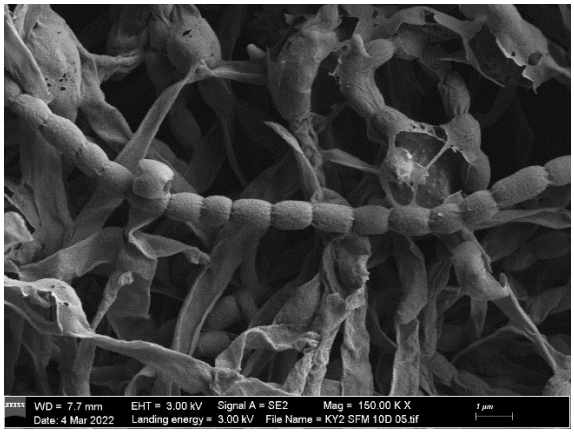


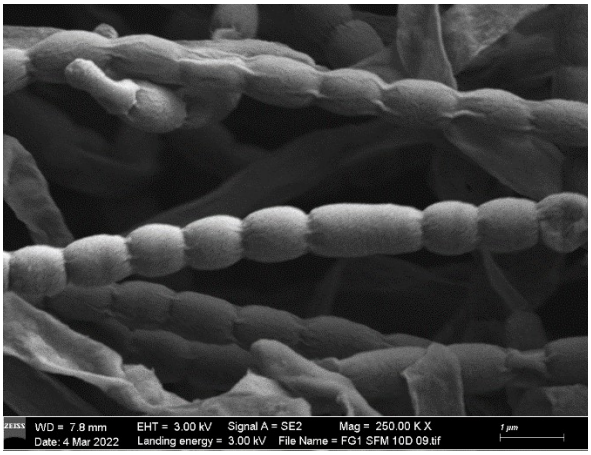




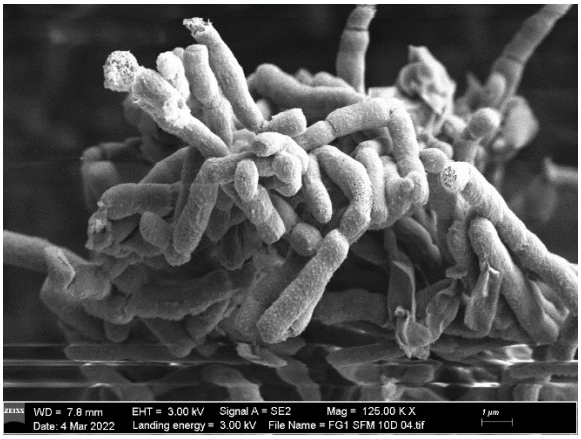
7.1.10 *Streptomyces* KY4

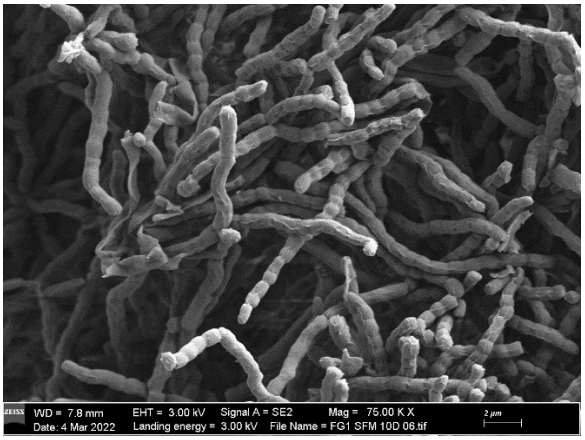


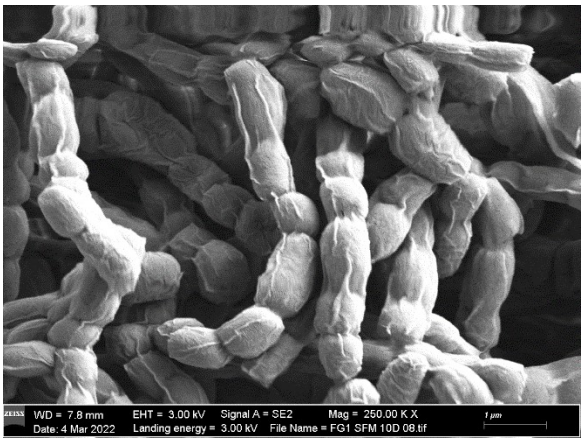




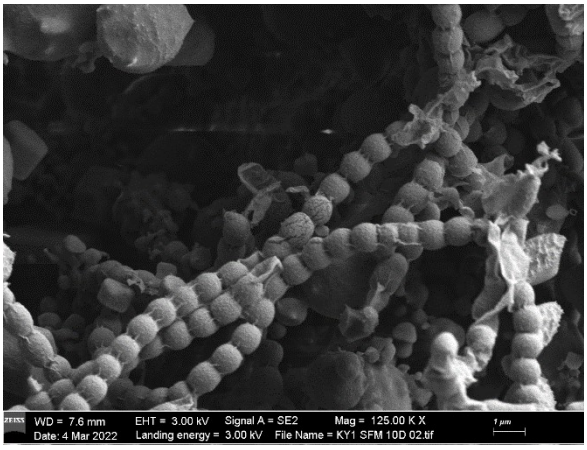
7.1.11 *Streptomyces* FG1

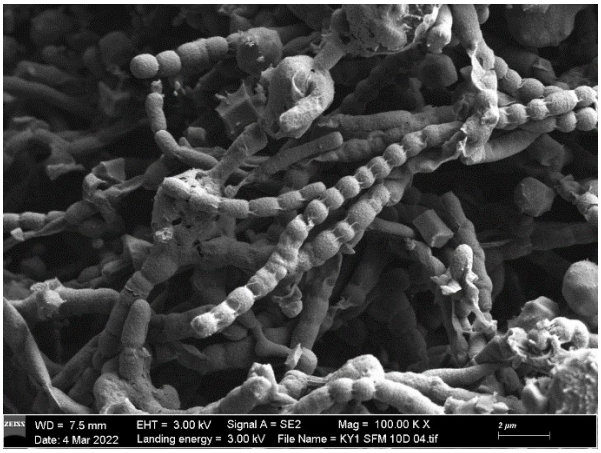


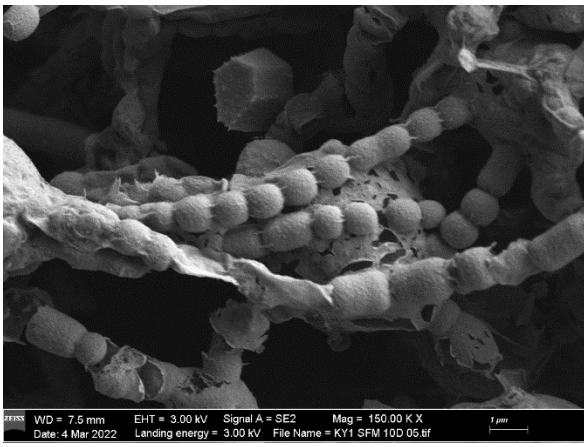


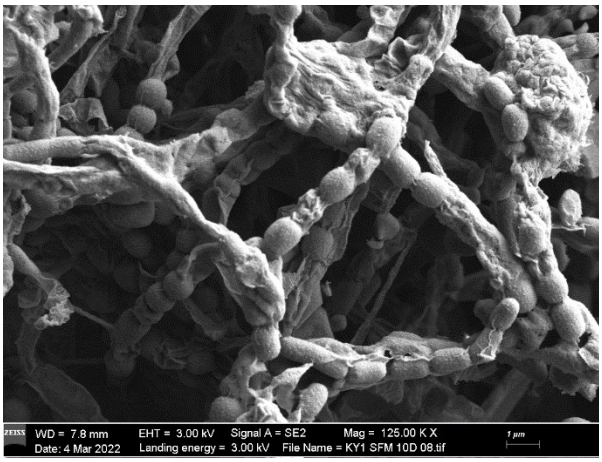


7.1.12 *Streptomyces* KY1

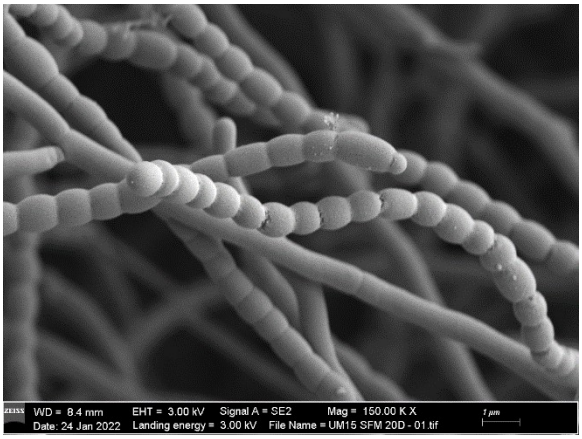


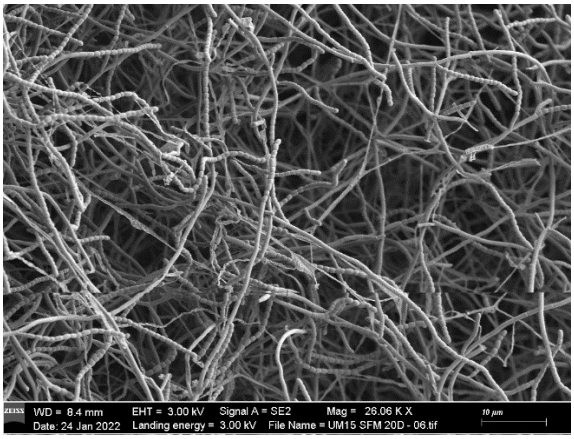


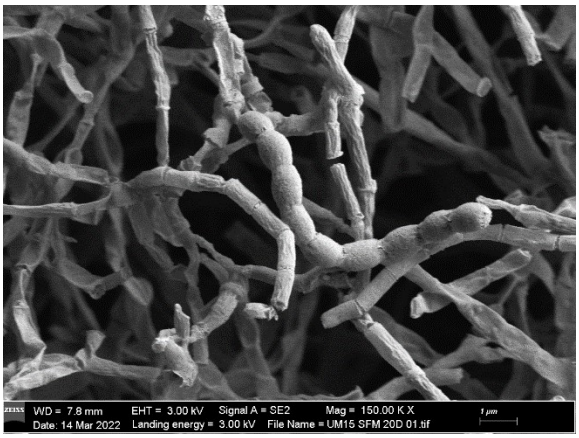


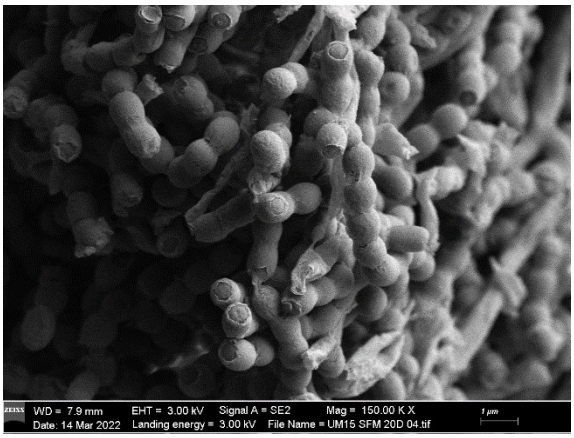


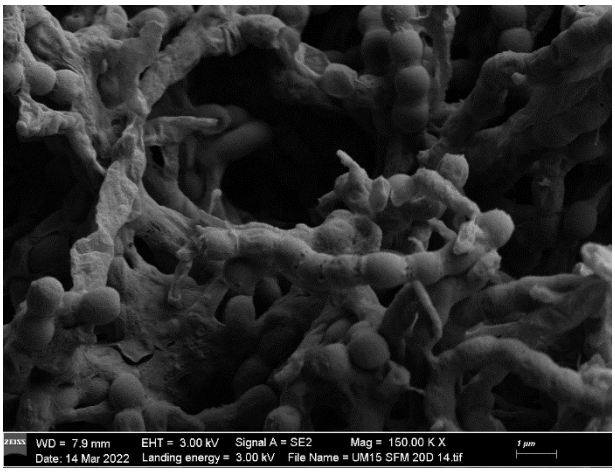
7.1.13 *Amycolatopsis* UM15



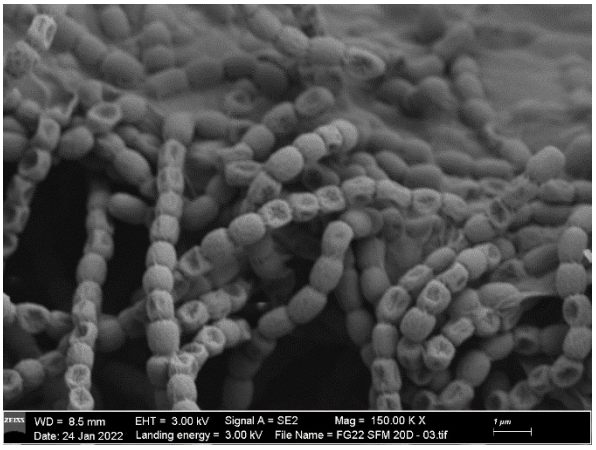


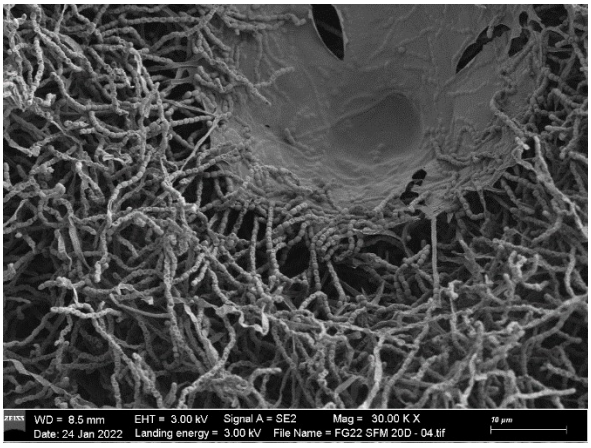


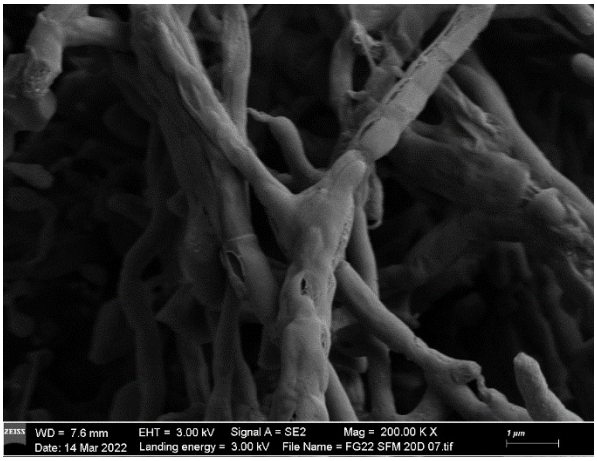


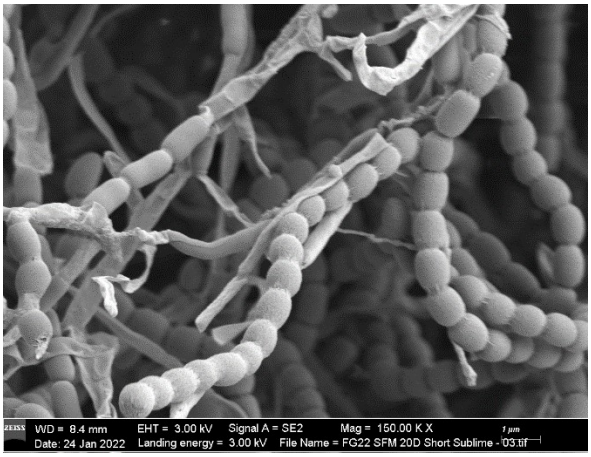


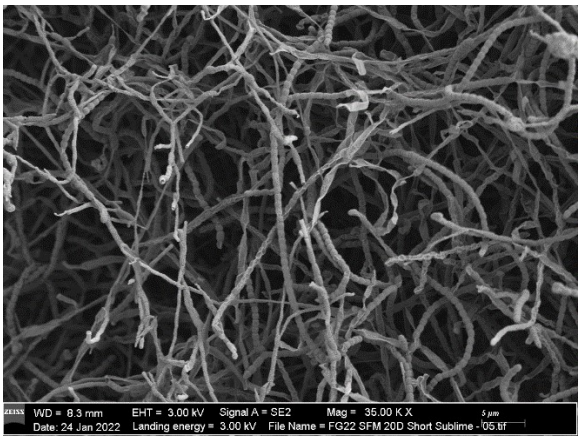
7.1.14 *Amycolatopsis* FG22



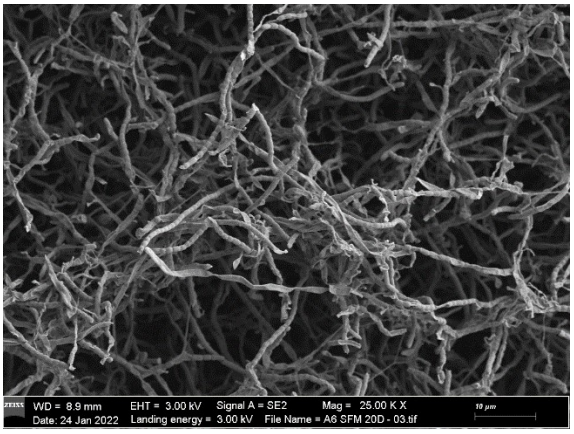


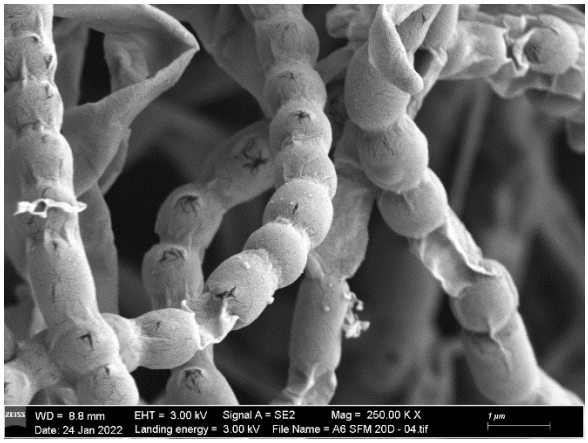


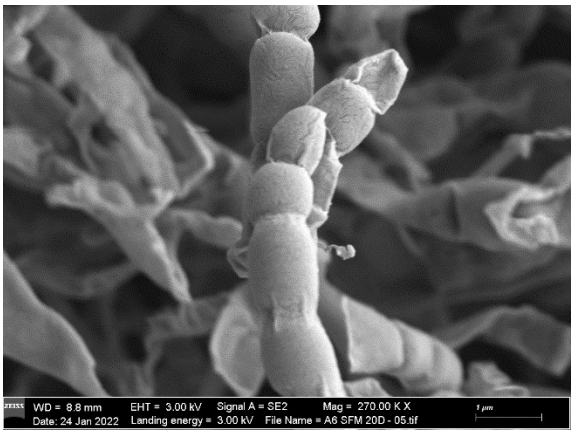


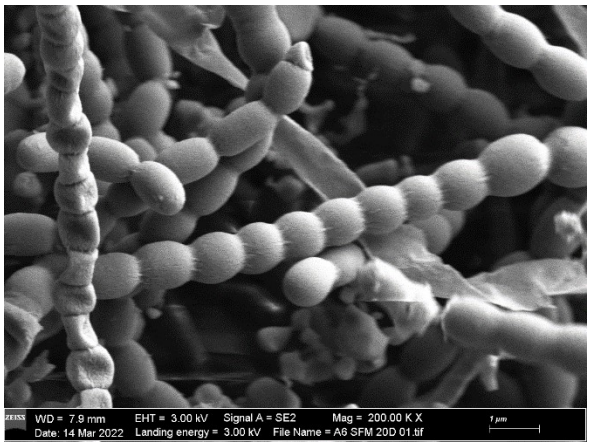


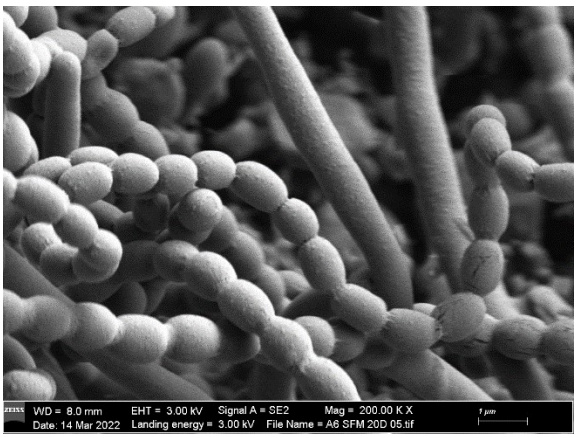
7.1.15 *Agrococcus* A6

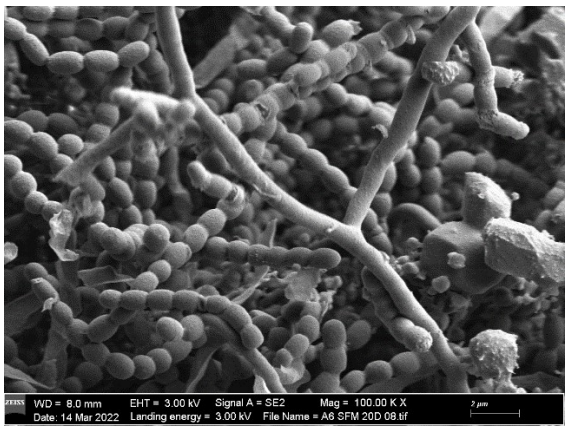




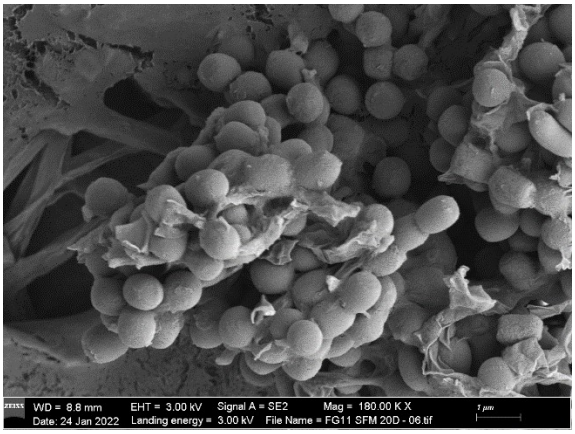


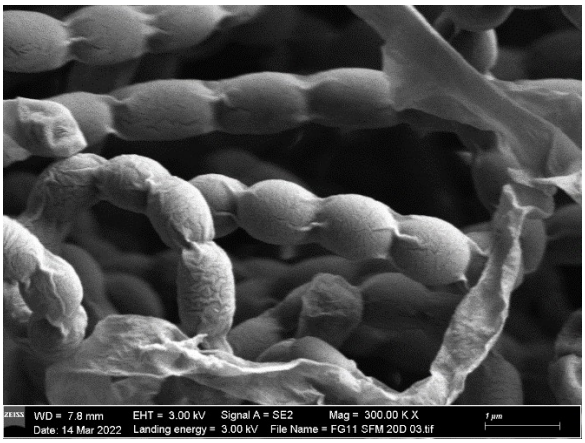


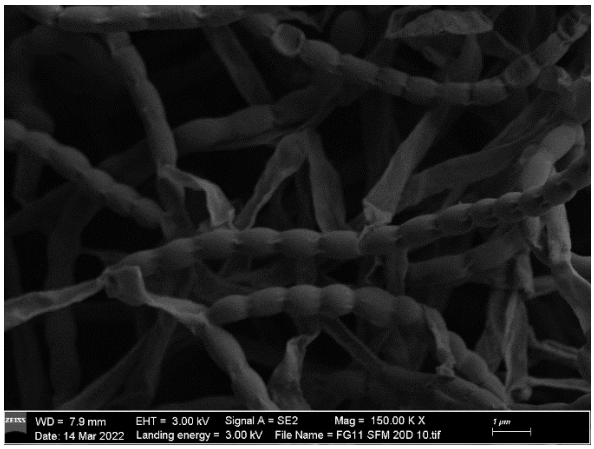




7.1.16 *Tsukamurella* FG11







7.2 AntiSMASH Analysis Results

7.2.1 *Pseudonocardia* UM4

Region	BGC Type	From (bp)	To (bp)	Most Similar Known Cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1.1	NAPAA	269,572	302,334	ikarugamycin	NRP + Polyketide:Iterative type I	8%
Region 1.2	oligosaccharide	406,909	452,926			
Region 1.3	ectoine	976,192	986,587	ectoine	Other	100%
Region 1.4	ranthipeptide	1,069,797	1,091,381			
Region 1.5	RiPP-like	1,441,999	1,450,328			
Region 1.6	terpene	2,497,869	2,518,468	carotenoid	Terpene	27%
Region 1.7	NAPAA,NRPS	2,849,762	2,922,554	scabichelin	NRP	60%
Region 1.8	terpene	3,144,656	3,165,903	SF2575	Polyketide:Type II + Saccharide:Hybrid/tailoring	6%
Region 1.9	NAPAA	3,424,982	3,459,469	streptobactin	NRP	11%
Region 1.10	T1PKS	3,696,145	3,837,868	nystatin-like Pseudonocardia polyene	Polyketide	100%
Region 1.11	redox-cofactor	3,962,928	3,985,042	lankacidin C	NRP + Polyketide	13%

Region 1.12	RiPP-like	4,936,905	4,947,726			
Region 2.1	NAPAA	60,484	94,980	glycopeptidolipid	NRP	10%
Region 2.2	lassopeptide	125,987	148,523			

7.2.2 *Pseudonocardia* UM14

Region	BGC Type	From (bp)	To (bp)	Most Similar Known Cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1	terpene	187,288	206,435	SF2575	Polyketide:Type II + Saccharide:Hybrid/tailoring	6%
Region 2	RiPP-like	432,504	442,769			
Region 3	terpene	459,375	481,921	hopene	Terpene	38%
Region 4	NRPS	625,288	676,810	coelibactin	NRP	90%*
Region 5	betalactone	701,032	732,402	frenolicin B	Polyketide	13%
Region 6	hglE-KS	823,081	870,097	dutomycin	Polyketide	6%
Region 7	betalactone	960,428	982,218			
Region 8	betalactone	1,324,529	1,350,599	nocathiacin	RiPP:Thiopeptide	4%
Region 9	NRPS	1,865,407	1,918,992	amychelin	NRP	18%
Region 10	RiPP-like	2,236,202	2,247,179			
Region 11	NRPS	2,484,860	2,554,050	scabichelin	NRP	60%
Region 12	ranthipeptide	2,727,655	2,749,313			
Region 13	ectoine	2,815,072	2,825,482	ectoine	Other	100%
Region 14	NAPAA	3,058,325	3,098,281			
Region 15	oligosaccharide	3,555,127	3,599,474	BE-7585A	Polyketide	7%
Region 16	betalactone	3,658,135	3,679,195	A-47934	NRP:Glycopeptide	8%
Region 17	T1PKS,NRPS-like	4,587,486	4,648,489	macbecin / macbecin II	Polyketide	47%
Region 18	betalactone	5,115,075	5,142,347	clavulanic acid	Other:Non-NRP beta-lactam	8%
Region 19	terpene	6,286,734	6,307,642	carotenoid	Terpene	27%

*Exact similarity unknown as manual review determined the similarity generated by antiSMASH is likely over-estimated.

7.2.3 *Pseudonocardia* UM9

Region	BGC Type	From (bp)	To (bp)	Most Similar Known Cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 2.1	redox-cofactor	27,367	49,502	lankacidin C	NRP + Polyketide	13%
Region 2.2	T1PKS,NRPS	475,400	523,365	yersiniabactin	NRP + Polyketide	4%
Region 2.3	RiPP-like	1,091,657	1,098,764			
Region 2.4	ranthipeptide	1,896,357	1,917,941			
Region 2.5	ectoine	2,005,221	2,015,616	ectoine	Other	100%
Region 2.6	NAPAA	2,801,191	2,833,554			
Region 2.7	T1PKS,NRPS-like	3,242,306	3,305,168	coelimycin P1	Polyketide:Modular type I	8%
Region 2.8	T1PKS,NRPS-like,NRPS	3,307,459	3,444,808	microansamycin	Polyketide	46%
Region 2.9	NAPAA	3,712,061	3,744,048	streptobactin	NRP	11%
Region 2.10	terpene	4,003,181	4,022,203	SF2575	Polyketide:Type II + Saccharide:Hybrid/tailoring	6%
Region 2.11	NRPS,NAPAA	4,262,865	4,339,850	scabichelin	NRP	60%
Region 2.12	terpene	4,626,507	4,647,502	isorenieratene	Terpene	28%
Region 2.13	RiPP-like	5,913,766	5,924,647			
Region 3.1	NRPS	1	15,835			
Region 4.1	lassopeptide	26,046	48,606			
Region 4.2	linaridin	86,748	113,409			

7.2.4 *Pseudonocardia* P1

Region	BGC Type	From (bp)	To (bp)	Most Similar Known Cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1.1	T1PKS	129,841	272,375	nystatin-like *Pseudonocardia* polyene	Polyketide	100%
Region 1.2	NAPAA,RiPP-like	666,353	705,357	streptobactin	NRP	11%
Region 1.3	redox-cofactor	836,501	856,239	lankacidin C	NRP + Polyketide	20%
Region 1.4	redox-cofactor	1,032,940	1,055,033	lankacidin C	NRP + Polyketide	13%
Region 1.5	terpene	1,420,179	1,440,732	isorenieratene	Terpene	28%
Region 1.6	betalactone	1,768,356	1,792,056			
Region 1.7	NRPS	1,842,376	1,896,570	mirubactin	NRP	21%
Region 1.8	other	3,163,130	3,203,642	thiocoraline	NRP:Cyclic depsipeptide	5%
Region 1.9	NAPAA	3,390,676	3,424,602	tomaymycin	NRP	11%
Region 1.10	oligosaccharide	3,515,138	3,562,121			
Region 1.11	ectoine	4,085,743	4,096,138	ectoine	Other	100%
Region 1.12	NRPS	4,160,564	4,207,497	mirubactin	NRP	50%
Region 1.13	ranthipeptide	4,215,020	4,236,614			
Region 1.14	NAPAA	4,242,229	4,278,403	stenothricin	NRP:Cyclic depsipeptide	13%
Region 1.15	RiPP-like	4,623,262	4,634,113			
Region 1.16	NAPAA	5,211,754	5,245,989			
Region 1.17	betalactone	5,948,208	5,979,405			
Region 1.18	NRPS	6,021,868	6,075,682	scabichelin	NRP	40%
Region 1.19	terpene	6,301,619	6,322,866	SF2575	Polyketide:Type II + Saccharide:Hybrid/tailoring	6%
Region 2.1	betalactone	171,554	233,467	xantholipin	Polyketide	4%

7.2.5 *Streptomyces* KY2

Region	BGC Type	From (bp)	To (bp)	Most Similar Known Cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1	transAT-PKS,NRPS,NRPS-like	1	92,281	phthoxazolin	NRP + Polyketide	18%
Region 2	NRPS,T1PKS,lanthipeptide-class-v	170,387	288,694	coelibactin	NRP	45%
Region 3	CDPS	307,042	327,469	naseseazine C / C3-aryl pyrroloindolines	Other	66%
Region 4	NRPS	495,329	543,868	actinonin	NRP	100%
Region 5	terpene	680,598	704,307	hopene	Terpene	69%
Region 6	betalactone	773,141	803,166			
Region 7	NRPS	873,521	1,019,482	surugamide A / surugamide D	NRP	57%
Region 8	terpene	1,086,291	1,107,111	fluostatins M-Q	Polyketide	4%
Region 9	NRPS	1,182,870	1,235,994	kedarcidin	Polyketide:Iterative type I + Polyketide:Enediyne type I	4%
Region 10	NRPS	1,238,209	1,280,683	qinichelins	NRP	16%
Region 11	RiPP-like	1,456,782	1,468,108			
Region 12	NRPS	1,672,154	1,726,653	clavulanic acid	Other:Non-NRP beta-lactam	8%
Region 13	siderophore	1,876,448	1,890,314	ficellomycin	NRP	3%
Region 14	NRPS-like	2,742,946	2,784,637	CDA1b / CDA2a / CDA2b / CDA3a / CDA3b / CDA4a / CDA4b	NRP:Ca+-dependent lipopeptide	12%
Region 15	ectoine	2,908,973	2,918,413	kosinostatin	NRP + Polyketide	13%
Region 16	T2PKS	2,935,488	3,008,087	murayaquinone	Polyketide	12%
Region 17	lanthipeptide-class-iii	3,671,029	3,693,836	chrysomycin	Polyketide	5%
Region 18	NRPS-like	5,123,983	5,166,169	bottromycin A2	RiPP:Bottromycin	45%

Region 19	NRPS	5,307,892	5,392,990	ulleungmycin	NRP	13%
Region 20	terpene	5,906,427	5,927,866	daptomycin	NRP	3%
Region 21	lanthipeptide-class-i	6,345,108	6,369,472			
Region 22	ectoine	6,510,271	6,520,675	ectoine	Other	100%
Region 23	siderophore	6,580,279	6,592,084	desferrioxamine E	Other	100%
Region 24	NRPS	6,787,742	6,852,042	cyclomarin D	NRP	13%
Region 25	CDPS	7,295,541	7,314,198	naseseazine C / C3-aryl pyrroloindolines	Other	100%
Region 26	terpene	7,388,367	7,417,952	carotenoid	Terpene	54%
Region 27	NRPS	7,773,898	7,834,487	ulleungmycin	NRP	8%
Region 28	NRPS-like,NRPS	7,908,532	7,960,279	vazabitide A	NRP	8%
Region 29	CDPS,T1PKS,NRPS-like	8,008,683	8,163,329	candicidin	Polyketide	76%
Region 30	NRPS	8,312,045	8,364,303	malonomycin	NRP + Polyketide	38%
Region 31	NRPS	8,428,638	8,492,188	ulleungmycin	NRP	11%
Region 32	lassopeptide	8,553,780	8,575,988	A-500359 A / A-500359 B	NRP	5%

7.2.6 *Streptomyces* B2

Region	BGC Type	From (bp)	To (bp)	Most Similar Known Cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1.1	lanthipeptide-class-iii	483,401	506,094	AmfS	RiPP:Lanthipeptide	100%
Region 1.2	ectoine	1,096,704	1,107,102	ectoine	Other	100%
Region 1.3	NRPS,ladderane	1,671,229	1,728,659	CDA1b / CDA2a / CDA2b / CDA3a / CDA3b / CDA4a / CDA4b	NRP:Ca+-dependent lipopeptide	27%
Region 1.4	RRE-containing	2,176,167	2,197,330			
Region 1.5	NRPS,T1PKS	2,286,836	2,361,830	sporolide A / sporolide B	NRP + Polyketide:Enediyne type I	51%
Region 1.6	terpene	4,016,533	4,038,245	geosmin	Terpene	100%
Region 1.7	butyrolactone	4,266,704	4,277,708			
Region 1.8	T1PKS,NRPS	4,406,391	4,455,308	SGR PTMs	NRP + Polyketide	100%
Region 1.9	terpene	4,828,131	4,846,864	julichrome Q3-3 / julichrome Q3-5	Polyketide	22%
Region 1.10	siderophore	5,392,441	5,407,874			
Region 1.11	NRPS	5,622,783	5,683,190	scabichelin	NRP	100%
Region 1.12	RiPP-like	5,717,099	5,728,388			
Region 1.13	NRPS,T3PKS	5,813,938	5,877,360	A40926	NRP:Glycopeptide + Saccharide:Hybrid/tailoring	12%
Region 1.14	terpene	6,205,389	6,230,449	hopene	Terpene	76%
Region 1.15	hglE-KS,T1PKS	6,286,622	6,339,711	ambactin	NRP	25%
Region 1.16	NRPS	6,554,406	6,594,222	paenibactin	NRP	83%
Region 1.17	NRPS	6,704,205	6,756,358	coelibactin	NRP	100%
Region 1.18	melanin	6,922,227	6,932,640	istamycin	Saccharide	4%
Region 1.19	terpene	7,008,367	7,033,107	isorenieratene	Terpene	85%

7.2.7 *Streptomyces* FG4

Region	BGC Type	From (bp)	To (bp)	Most Similar Known Cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1.1	NAPAA	73,086	108,946	stenothricin	NRP:Cyclic depsipeptide	13%
Region 1.2	NRPS	219,751	280,813	scabichelin	NRP	100%
Region 1.3	phenazine	348,808	369,251	lomofungin	Other	39%
Region 1.4	thiopeptide	585,796	624,202	natamycin	Polyketide	9%
Region 1.5	T3PKS	684,544	724,089	herboxidiene	Polyketide	7%
Region 1.6	ectoine	1,373,768	1,384,172	ectoine	Other	100%
Region 1.7	melanin	2,188,803	2,199,282	istamycin	Saccharide	4%
Region 1.8	siderophore	2,271,589	2,281,402	desferrioxamin B / desferrioxamine E	Other	83%
Region 1.9	T2PKS	2,858,638	2,931,143	spore pigment	Polyketide	83%
Region 1.10	terpene	4,336,168	4,356,565	albaflavenone	Terpene	100%
Region 1.11	ladderane,furan,butyrolactone	4,568,660	4,609,835	colabomycin E	Polyketide:Type II	20%
Region 1.12	siderophore	4,918,083	4,928,560			
Region 1.13	butyrolactone	4,937,306	4,948,292	kinamycin	Polyketide	11%
Region 1.14	NRPS,betalactone	4,999,955	5,083,825	WS9326	NRP	12%
Region 1.15	RiPP-like	5,191,463	5,202,015			
Region 1.16	terpene	5,214,820	5,234,044	geosmin	Terpene	100%
Region 1.17	siderophore	5,316,819	5,329,949	grincamycin	Polyketide:Type II + Saccharide:Hybrid/tailoring	8%
Region 1.18	T1PKS	5,393,354	5,435,053	kanamycin	Saccharide	61%
Region 1.19	terpene	5,597,661	5,624,344	hopene	Terpene	92%
Region 1.20	T3PKS,NRPS	5,918,493	5,994,781	marformycin A / marformycin B / marformycin C / marformycin D / marformycin E / marformycin F	NRP	12%

Region 1.21	T1PKS	6,114,747	6,161,055	ansamitocin P-3	Polyketide	4%
Region 1.22	NRPS	6,193,478	6,252,048	cadaside A / cadaside B	NRP	19%
Region 1.23	RiPP-like	6,303,456	6,313,671	informatipeptin	RiPP:Lanthipeptide	42%
Region 1.24	LAP,thiopeptide	6,492,186	6,526,853	dutomycin	Polyketide	4%

7.2.8 *Streptomyces* FG7

Region	BGC Type	From (bp)	To (bp)	Most Similar Known Cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1	T2PKS,butyrolactone	219,479	291,426	kinamycin	Polyketide	45%
Region 2	T3PKS	389,614	424,274	alkylresorcinol	Polyketide	100%
Region 3	NRPS,NAPAA	436,469	510,618	stenothricin	NRP:Cyclic depsipeptide	22%
Region 4	T3PKS	537,933	578,985	herboxidiene	Polyketide	7%
Region 5	ectoine	1,177,085	1,187,489	ectoine	Other	100%
Region 6	lassopeptide	1,807,736	1,830,215	kanamycin	Saccharide	1%
Region 7	melanin	2,053,946	2,064,512	istamycin	Saccharide	4%
Region 8	siderophore	2,130,112	2,140,873	desferrioxamin B / desferrioxamine E	Other	83%
Region 9	butyrolactone	3,328,007	3,347,791	scleric acid	NRP	17%
Region 10	T2PKS	3,426,860	3,499,365	spore pigment	Polyketide	83%
Region 11	terpene	4,188,865	4,209,433	albaflavenone	Terpene	100%
Region 12	siderophore	4,755,472	4,765,904			
Region 13	NRPS	4,831,656	4,884,370	rimosamide	NRP	21%
Region 14	NRPS	4,926,297	4,974,979			
Region 15	RiPP-like	5,007,429	5,018,572			
Region 16	terpene	5,030,755	5,050,684	geosmin	Terpene	100%
Region 17	NRPS,siderophore	5,113,093	5,172,210	sarpeptin A / sarpeptin B	NRP	25%
Region 18	terpene	5,378,937	5,405,682	hopene	Terpene	92%
Region 19	NRPS-like,NRPS,T1PKS	5,467,503	5,530,625	WS9326	NRP	10%

7.2.9 *Streptomyces* A7

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1	T1PKS	43,995	246,106	lobophorin A	Polyketide	93%
Region 2	terpene	271,642	291,317	ebelactone	Polyketide	5%
Region 3	T3PKS	314,910	354,935	germicidin	Other	100%
Region 4	indole	501,010	522,137	5-isoprenylindole-3-carboxylate β-D-glycosyl ester	Other	28%
Region 5	terpene	573,500	595,890	carotenoid	Terpene	54%
Region 6	amglyccycl	775,593	796,831	β-D-galactosylvalidoxylamine-A	Saccharide	22%
Region 7	T3PKS	1,029,687	1,070,799	herboxidiene	Polyketide	8%
Region 8	NRPS	1,143,584	1,196,081	rimosamide	NRP	21%
Region 9	T1PKS	1,280,740	1,355,407	butyrolactol A	Polyketide	86%
Region 10	ectoine	1,779,678	1,790,076	ectoine	Other	100%
Region 11	melanin	2,768,419	2,779,045	melanin	Other	60%
Region 12	lassopeptide	2,840,550	2,863,071	SSV-2083	RiPP:Lanthipeptide	36%
Region 13	siderophore	2,876,755	2,887,599	desferrioxamin B / desferrioxamine E	Other	83%
Region 14	phenazine	3,597,729	3,618,214			
Region 15	LAP,thiopeptide	3,835,567	3,868,172	diazepinomicin	Terpene	7%
Region 16	lanthipeptide-class-ii	4,393,289	4,421,476	SBI-06990 A1 / SBI-06989 A2	RiPP:Lanthipeptide	50%
Region 17	T1PKS,NRPS-like	5,292,729	5,354,534	naphthomycin A	Polyketide	53%
Region 18	betalactone	5,472,349	5,497,899	julichrome Q3-3 / julichrome Q3-5	Polyketide	22%
Region 19	terpene	5,499,052	5,518,863	albaflavenone	Terpene	100%
Region 20	T2PKS	5,569,626	5,642,180	spore pigment	Polyketide	66%
Region 21	siderophore	6,083,968	6,094,196			

Region 22	NRPS,NRPS-like,T1PKS	6,179,358	6,291,036	friulimicin A / friulimicin B / friulimicin C / friulimicin D	NRP	75%
Region 23	NRPS	6,298,739	6,347,832	telomycin	NRP	29%
Region 24	NRPS,T1PKS	6,392,703	6,441,126	xiamycin A	Terpene	72%
Region 25	RiPP-like	6,491,263	6,502,099			
Region 26	terpene	6,533,105	6,554,762	geosmin	Terpene	100%
Region 27	lanthipeptide-class-i	6,610,414	6,634,552	tobramycin	Saccharide	5%
Region 28	siderophore	6,735,567	6,748,741			
Region 29	NRPS	6,781,203	6,824,564	diisonitrile antibiotic SF2768	NRP	55%
Region 30	PKS-like	6,856,849	6,897,874	caboxamycin	NRP + Polyketide	60%
Region 31	NRPS,nucleoside	6,905,156	6,946,207	nogalamycin	Polyketide	40%
Region 32a	terpene	7,272,301	7,286,111	hopene	Terpene	92%
Region 32b	NRPS	7,301,863	7,327,219	coelibactin	NRP	100%
Region 33a	NRPS	7,376,378	7,400,751	mirubactin	NRP	78%
Region 33b	T1PKS	7,421,725	7,494,125	divergolide A / divergolide B / divergolide C / divergolide D	Polyketide:Modular type I	100%
Region 34	NRPS	7,538,435	7,601,057	cyclomarin D	NRP	8%
Region 35	terpene	7,824,810	7,845,838	versipelostatin	Polyketide	5%
Region 36	RiPP-like	7,858,260	7,868,475	informatipeptin	RiPP:Lanthipeptide	42%
Region 37	NRPS	8,073,065	8,123,993	coelichelin	NRP	100%
Region 38	T1PKS	8,261,305	8,349,864	funisamine	Polyketide	25%

7.2.10 *Streptomyces* KY4

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1.1	NRPS,T1PKS,other,NRPS-like	312,879	415,395	aurantimycin A	NRP + Polyketide	39%
Region 1.2	NRPS	489,463	540,202	coelibactin	NRP	100%
Region 1.3	indole	793,605	814,732	5-isoprenylindole-3-carboxylate β-D-glycosyl ester	Other	28%
Region 1.4	T3PKS	1,027,507	1,068,058	germicidin	Other	100%
Region 1.5	T1PKS	1,158,778	1,244,904	divergolide A / divergolide B / divergolide C / divergolide D	Polyketide:Modular type I	41%
Region 1.6	T3PKS	1,545,853	1,583,224	herboxidiene	Polyketide	8%
Region 1.7	ectoine	2,263,404	2,273,802	ectoine	Other	100%
Region 1.8	T1PKS	2,936,414	3,018,717	carrimycin	Polyketide	20%
Region 1.9	melanin	3,380,870	3,391,433	melanin	Other	60%
Region 1.10	siderophore	3,477,163	3,487,970	desferrioxamin B / desferrioxamine E	Other	83%
Region 1.11	RRE-containing	5,770,524	5,790,279	naphthomycin A	Polyketide	9%
Region 1.12	terpene	5,917,926	5,938,620	albaflavenone	Terpene	100%
Region 1.13	T2PKS	5,986,474	6,059,016	spore pigment	Polyketide	66%
Region 1.14	siderophore	6,501,327	6,512,480			
Region 1.15	RiPP-like	6,792,232	6,801,999			
Region 1.16	terpene	6,823,425	6,843,503	geosmin	Terpene	100%

Region 1.17	siderophore	7,009,764	7,022,957			
Region 1.18	NRPS-like,NRPS	7,140,306	7,187,823	thaxteramide C	NRP	7%
Region 1.19	lanthipeptide-class-iii	7,470,311	7,493,118	SapB	RiPP:Lanthipeptide	100%
Region 1.20	terpene	7,568,420	7,594,226	hopene	Terpene	100%
Region 1.21	RiPP-like	7,965,893	7,976,108	informatipeptin	RiPP:Lanthipeptide	42%
Region 1.22	NRPS	8,236,768	8,287,753	coelichelin	NRP	100%
Region 1.23	T3PKS	8,500,917	8,542,098	alkylresorcinol	Polyketide	100%
Region 1.24	terpene	8,564,415	8,589,569	isorenieratene	Terpene	100%
Region 1.25	hglE-KS,T1PKS	8,594,918	8,647,981	diazaquinomycin H / diazaquinomycin J	Other	4%
Region 1.26	lanthipeptide-class-i	8,729,670	8,754,716			
Region 3.1	T1PKS	1	53,945	salinilactam	Polyketide	48%
Region 3.2	butyrolactone,redox-cofactor,T1PKS	57,878	133,130	griseoviridin / fijimycin A	NRP:Cyclic depsipeptide + Polyketide:Trans-AT type I	16%

7.2.11 *Streptomyces* FG1

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1	T1PKS,NRPS,LAP,thiopeptide	109,317	160,925	lactazole	RiPP:Thiopeptide	33%
Region 2	terpene	175,414	199,875	isorenieratene	Terpene	100%
Region 3	NRPS,T3PKS	310,457	426,449	herboxidiene	Polyketide	12%
Region 4	melanin	462,534	470,286	melanin	Other	100%
Region 5	NRPS	479,558	536,631	valinomycin / montanastatin	NRP + Saccharide:Hybrid/tailoring	91%
Region 6	oligosaccharide,T2PKS,other,PKS-like,NRPS	576,783	702,314	cinerubin B	Polyketide:Type II	74%
Region 7	RiPP-like,T1PKS,NRPS	745,493	800,173	SGR PTMs	NRP + Polyketide	100%
Region 8	T1PKS	838,469	935,547	bafilomycin B1	Polyketide:Modular type I	100%
Region 9	thioamide-NRP	939,609	989,063	cadaside A / cadaside B	NRP	19%
Region 10	terpene	1,022,485	1,048,698	hopene	Terpene	69%
Region 11	NRPS,NRPS-like	1,457,602	1,503,182	salinomycin	Polyketide:Modular type I	12%
Region 12	RiPP-like	1,645,773	1,657,179			
Region 13	NRPS	1,759,651	1,800,379	FD-594	Polyketide	6%
Region 14	siderophore	2,021,459	2,033,660	ficellomycin	NRP	3%
Region 15	amglyccycl	2,412,705	2,433,907	acarbose	Saccharide	14%
Region 16	terpene	2,446,396	2,465,908			
Region 17	lanthipeptide-class-iii	2,797,068	2,819,104	AmfS	RiPP:Lanthipeptide	100%
Region 18	lassopeptide	3,642,475	3,665,190	keywimysin	RiPP	100%
Region 19	T2PKS	3,717,234	3,789,725	prejadomycin / rabelomycin / gaudimycin C / gaudimycin D / UWM6 / gaudimycin A	Polyketide:Type II + Saccharide:Hybrid/tailoring	25%

Region 20	betalactone	4,387,745	4,415,675	divergolide A / divergolide B / divergolide C / divergolide D	Polyketide:Modular type I	6%
Region 21	butyrolactone,ectoine	4,575,110	4,589,322	showdomycin	Other	47%
Region 22	NRPS	4,906,252	4,960,663	atratumycin	NRP	5%
Region 23	ectoine	5,168,825	5,177,826	ectoine	Other	50%
Region 24	thiopeptide,LAP	5,184,496	5,216,991			
Region 25	NRPS-like	5,452,779	5,495,309	bottromycin A2	RiPP:Bottromycin	39%
Region 26	siderophore	5,574,745	5,586,523	desferrioxamin B	Other	100%
Region 27	lanthipeptide-class-iii,lanthipeptide-class-ii	5,685,692	5,717,281			
Region 28	ectoine	6,658,118	6,668,516	ectoine	Other	100%
Region 29	terpene	7,115,939	7,135,323	steffimycin D	Polyketide:Type II + Saccharide:Hybrid/tailoring	19%
Region 30	T3PKS	7,613,631	7,654,749	herboxidiene	Polyketide	6%
Region 31	NRPS,melanin	7,698,832	7,748,482	coelichelin	NRP	81%
Region 32	NRPS,transAT-PKS,T1PKS,PKS-like	7,772,075	7,880,076	streptobactin	NRP	94%
Region 33	terpene	7,883,529	7,905,742	geosmin	Terpene	100%
Region 34	butyrolactone	7,932,925	7,943,794	coelimycin P1	Polyketide:Modular type I	16%
Region 35	arylpolyene,NRPS-like	7,952,992	7,996,134	atratumycin	NRP	7%

7.2.12 *Streptomyces* KY1

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1.1	T1PKS,T3PKS	1	93,735	herboxidiene	Polyketide	15%
Region 1.2	terpene,RiPP-like	165,814	193,124	isorenieratene	Terpene	85%
Region 1.3	NRPS-like	217,308	260,369	indigoidine	NRP	80%
Region 1.4	ectoine	1,003,823	1,014,221	ectoine	Other	100%
Region 1.5	siderophore	1,929,414	1,940,578	desferrioxamin B	Other	100%
Region 1.6	T2PKS	3,346,579	3,419,037	julichrome Q3-3 / julichrome Q3-5	Polyketide	59%
Region 1.7	lanthipeptide-class-iii	3,716,840	3,739,419	AmfS	RiPP:Lanthipeptide	80%
Region 1.8	thiopeptide,LAP	4,045,854	4,078,349	fluostatins M-Q	Polyketide	4%
Region 1.9	terpene	4,577,400	4,597,247	julichrome Q3-3 / julichrome Q3-5	Polyketide	25%
Region 1.10	terpene	4,930,870	4,951,210	geosmin	Terpene	100%
Region 1.11	siderophore	5,201,262	5,216,362	ficellomycin	NRP	5%
Region 1.12	RiPP-like	5,542,863	5,552,994			
Region 1.13	RiPP-like	5,946,649	5,956,864			
Region 1.14	terpene	6,039,251	6,065,806	hopene	Terpene	76%
Region 1.15	T1PKS,NRPS	6,104,171	6,153,578	SGR PTMs	NRP + Polyketide	100%
Region 2.1	lassopeptide	11,067	33,595			

Region 3.1	T1PKS,NRPS-like,NRPS	9,117	108,152	antimycin	NRP:Cyclic depsipeptide + Polyketide:Modular type I	100%
Region 3.2	T1PKS,NRPS-like	108,583	230,490	candicidin	Polyketide	90%
Region 5.1	transAT-PKS	1	22,133	epothilone	NRP + Polyketide	42%

7.2.13 *Amycolatopsis* UM15

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1.1	NAPAA,betalactone, NRPS,redox-cofactor	63,294	145,287	mirubactin	NRP	78%
Region 1.2	terpene	212,104	236,884	hopene	Terpene	46%
Region 1.3	CDPS	282,475	303,158			
Region 1.4	NRPS	327,068	374,215	carbapenem MM4550	Other:Non-NRP beta-lactam	6%
Region 1.5	PKS-like	502,401	538,588	caboxamycin	NRP + Polyketide	50%
Region 1.6	terpene	664,256	683,473	SF2575	Polyketide:Type II + Saccharide:Hybrid/tailoring	4%
Region 1.7	siderophore	813,868	825,661	macrotetrolide	Polyketide	33%
Region 1.8	RiPP-like	850,809	859,173			
Region 1.9	terpene	1,194,106	1,214,705	isorenieratene	Terpene	42%
Region 1.10	T1PKS,butyrolactone	1,759,091	1,863,197	macrotermycins	Polyketide	96%
Region 1.11	T1PKS,thiopeptide,LAP,NRPS-like,redox-cofactor	2,013,527	2,111,098	caerulomycin A	NRP + Polyketide	36%
Region 1.12	hglE-KS	2,155,134	2,204,729	rifamorpholine A / rifamorpholine B / rifamorpholine C / rifamorpholine D / rifamorpholine E	Polyketide	11%
Region 1.13	NAPAA	2,600,402	2,634,370	stenothricin	NRP:Cyclic depsipeptide	13%
Region 1.14	RiPP-like	2,647,036	2,656,913			
Region 1.15	NAPAA	2,892,973	2,926,860			
Region 1.16	redox-cofactor	3,156,871	3,178,910	lankacidin C	NRP + Polyketide	20%
Region 1.17	betalactone	4,113,292	4,135,830			
Region 1.18	NRPS,thioamitides	4,255,418	4,372,255	amychelin	NRP	25%

Region 1.19	NRPS	4,387,780	4,431,628	A33853	Other	21%
Region 1.20	terpene	4,495,833	4,516,620			
Region 1.21	PKS-like	4,675,745	4,716,100			
Region 1.22	ladderane,thioamide -NRP,NRPS	5,749,507	5,821,797	ishigamide	NRP + Polyketide	61%
Region 1.23	terpene	5,938,752	5,961,052	geosmin	Terpene	100%
Region 1.24	lanthipeptide-class-iii,indole	6,942,575	6,971,652	fortimicin	Saccharide	9%
Region 1.25	ectoine	8,075,188	8,085,580	ectoine	Other	100%
Region 1.26	RiPP-like	8,572,762	8,583,559			
Region 1.27	T2PKS,PKS-like	8,801,202	8,873,657	cinerubin B	Polyketide:Type II	28%
Region 2.1	NRPS	1	30,822	amychelin	NRP	18%
Region 3.1	PKS-like,NRPS-like,terpene	211,531	278,189	platensimycin / platencin	Terpene	11%
Region 3.2	T1PKS	341,692	386,965	tiancimycin	Polyketide	16%
Region 3.3	lanthipeptide-class-iii	459,758	482,292	Ery-9 / Ery-6 / Ery-8 / Ery-7 / Ery-5 / Ery-4 / Ery-3	RiPP:Lanthipeptide	75%
Region 3.4	NRPS,terpene	483,583	595,901	2-methylisoborneol	Terpene	100%
Region 4.1	T1PKS	1	27,731	collismycin A	NRP + Polyketide:Modular type I	14%

7.2.14 *Amycolatopsis* FG22

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1	NRPS-like	1	29,350			
Region 2	NAPAA	476,423	508,212			
Region 3	NRPS	508,933	585,541	amychelin	NRP	25%
Region 4	thiopeptide	954,986	976,623			
Region 5	NRPS-like	1,052,099	1,094,645	meilingmycin	Polyketide	3%
Region 6	NRPS	1,676,060	1,715,695	avilamycin A / avilamycin C	Saccharide:Oligosaccharide	5%
Region 7	redox-cofactor	1,739,650	1,761,684	lankacidin C	NRP + Polyketide	20%
Region 8	thiopeptide,thioamitides ,other	2,109,995	2,174,861	neocarzinostatin	Polyketide:Iterative type I + Polyketide:Enediyne type I	13%
Region 9	T1PKS	2,209,713	2,253,082	neocarzinostatin	Polyketide:Iterative type I + Polyketide:Enediyne type I	8%
Region 10	ectoine	2,562,523	2,572,912	ectoine	Other	100%
Region 11	terpene,T1PKS	2,596,406	2,651,707	geosmin	Terpene	100%

Region 12	CDPS	2,719,521	2,740,222			
Region 13	lanthipeptide-class-iii	3,731,704	3,754,247	Ery-9 / Ery-6 / Ery-8 / Ery-7 / Ery-5 / Ery-4 / Ery-3	RiPP:Lanthipeptide	100%
Region 14	NRPS,T1PKS	4,535,482	4,578,602	chlortetracycline	Polyketide	5%
Region 15	arylpolyene	4,664,684	4,705,835	kinamycin	Polyketide	5%
Region 16	T2PKS	4,731,672	4,804,202	dactylocycline A	Polyketide	28%
Region 17	T1PKS,NRPS-like,NRPS,T3PKS	4,831,185	4,970,844	keratinimicin A / keratinimicin B / keratinimicin C / keratinimicin D	Other	88%
Region 18	thiopeptide,T1PKS	5,304,863	5,375,183	amycolamycin A / amycolamycin B	Polyketide	31%
Region 19	terpene	5,895,891	5,915,765	isorenieratene	Terpene	42%
Region 20	RiPP-like	6,246,758	6,257,573			
Region 21	terpene	6,450,850	6,470,771	vazabitide A	NRP	4%
Region 22	T1PKS	6,518,447	6,623,930	butyrolactol A	Polyketide	66%
Region 23	hglE-KS,T1PKS	6,658,419	6,708,913	rimosamide	NRP	14%
Region 24	PKS-like	6,979,641	7,020,774			

Region 25	lanthipeptide-class-i	7,182,931	7,207,996			
Region 26	T1PKS	7,274,846	7,319,121	kanamycin	Saccharide	1%
Region 27	NRPS	7,451,450	7,499,615	mirubactin	NRP	78%
Region 28	NRPS	7,598,155	7,658,902	albachelin	NRP	100%
Region 29	NRPS	7,961,372	8,018,850	surugamide A / surugamide D	NRP	9%
Region 30	NAPAA	8,043,702	8,077,222			
Region 31	terpene	8,113,233	8,134,372	2-methylisoborneol	Terpene	100%
Region 32	RRE-containing	8,168,662	8,189,759			
Region 33	lanthipeptide-class-ii	8,238,846	8,261,713			
Region 34	T1PKS,hglE-KS	8,703,152	8,795,410	rifamorpholine A / rifamorpholine B / rifamorpholine C / rifamorpholine D / rifamorpholine E	Polyketide	9%
Region 35	NAPAA	8,973,774	8,999,830	ansacarbamitocin A	Polyketide	4%

7.2.15 *Agrococcus* A6

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1	T1PKS	126,062	184,458	abyssomicin M / abyssomicin N / abyssomicin O / abyssomicin P / abyssomicin Q / abyssomicin R / abyssomicin S / abyssomicin T / abyssomicin U / abyssomicin V / abyssomicin W / abyssomicin X	Polyketide	6%
Region 2	ectoine	2,188,251	2,198,628	showdomycin	Other	23%
Region 3	betalactone	2,537,581	2,564,207	microansamycin	Polyketide	7%

7.2.16 *Tsukamurella* FG11

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1	NRPS	38,679	85,678	heterobactin A / heterobactin S2	NRP	63%
Region 2	NRPS	688,132	734,007	rhodochelin	NRP	33%
Region 3	terpene	1,344,160	1,362,390			
Region 4	T3PKS	1,549,907	1,591,112	kanamycin	Saccharide	1%
Region 5	RiPP-like	1,748,314	1,758,040			
Region 6	ectoine	2,399,195	2,409,584	ectoine	Other	75%
Region 7	thiopeptide,T1PKS,NRPS ,NRPS-like	2,528,821	2,638,024	neoantimycin	NRP + Polyketide	20%
Region 8	RiPP-like	2,891,314	2,900,915			
Region 9	betalactone	2,920,555	2,950,350			
Region 10	RiPP-like	3,086,325	3,097,122			
Region 11	terpene	3,588,478	3,609,296	sisomicin	Saccharide	5%

Region 12	butyrolactone	3,757,747	3,768,778			
Region 13	NRPS,T1PKS	3,769,759	3,854,497	amychelin	NRP	12%
Region 14	betalactone,NAPAA	4,533,879	4,589,585	aclacinomycin	Polyketide	8%

7.2.17 *Tsukamurella* E7

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 1.1	ectoine	84,582	94,971	ectoine	Other	75%
Region 1.2	T1PKS,NRPS,NRPS-like	183,013	255,110	9-methylstreptimidone	Polyketide:Modular type I	12%
Region 1.3	RiPP-like	515,093	524,002	lipopolysaccharide	Saccharide:Lipopolysaccharide	5%
Region 1.4	betalactone	542,709	572,469			
Region 1.5	RiPP-like	707,077	717,874			
Region 1.6	redox-cofactor	962,878	985,700			
Region 1.7	terpene	1,250,615	1,271,433	sisomicin	Saccharide	5%
Region 1.8	NRPS	1,331,910	1,381,444			
Region 1.9	T1PKS	1,466,231	1,511,348			
Region 1.10	NRPS,betalactone	2,166,265	2,215,767	ulleungmycin	NRP	5%

Region 1.11	NAPAA	2,404,536	2,438,471	cathomycin	Saccharide:Hybrid/tailoring + Other:Aminocoumarin	15%
Region 1.12	NRPS	2,493,402	2,541,960	mirubactin	NRP	28%
Region 1.13	linaridin	2,605,357	2,625,914			
Region 1.14	betalactone	2,827,878	2,856,484			
Region 2.1	terpene	971,104	992,153			
Region 2.2	T3PKS	1,184,287	1,225,519	kanamycin	Saccharide	1%
Region 2.3	RiPP-like	1,381,578	1,392,435			
Region 2.4	amglyccycl	1,651,289	1,672,515	acarbose	Saccharide	10%

7.2.18 *Jiangella* S1

Region	BGC Type	From (bp)	To (bp)	Most similar known cluster	Most Similar Known Cluster Type	Similarity to Most Similar Known Cluster
Region 2.1	redox-cofactor	97,518	129,378	LL-D49194α1 (LLD)	Polyketide	3%
Region 2.2	siderophore	1,293,978	1,309,350			
Region 2.3	lanthipeptide-class-v	1,897,149	1,939,325	prejadomycin / rabelomycin / gaudimycin C / gaudimycin D / UWM6 / gaudimycin A	Polyketide:Type II + Saccharide:Hybrid/tailoring	4%
Region 2.4	RRE-containing,thiopeptide,LAP	3,028,406	3,058,479			
Region 2.5	T3PKS	3,062,486	3,103,559	alkylresorcinol	Polyketide	100%
Region 2.6	betalactone	3,111,185	3,136,397	lipopolysaccharide	Saccharide:Lipopolysaccharide	5%
Region 4.1	lanthipeptide-class-i	408,102	434,615	keratinimicin A / keratinimicin B / keratinimicin C / keratinimicin D	Other	4%
Region 4.2	RiPP-like	1,658,479	1,669,279			

Region 4.3	NAGGN	1,864,525	1,879,605			
Region 4.4	NRPS-like	2,326,830	2,369,364			
Region 4.5	RRE-containing	3,380,709	3,398,339	ecumicin	NRP	10%
Region 4.6	lassopeptide,RRE-containing	3,427,083	3,449,524	vazabitide A	NRP	4%

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